University of Alberta

LONG-TERM EFFECTS OF PARITY ON BLOOD PRESSURE REGULATION

by

Vivek Dhawan ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Physiology

Edmonton, Alberta
Spring 2006

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ISBN: 0-494-13961-7
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ISBN: 0-494-13961-7

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ABSTRACT

Cardiovascular disease is the leading cause of death and disability in women (3). There are profound alterations in the cardiovascular system during pregnancy, the long-term effects of which are unknown. Human epidemiological studies suggest that multiparity (repeated pregnancies) increases the risk of cardiovascular disease. (10,98,117,153,155). It is difficult in epidemiological studies to control for potentially confounding factors such as socioeconomic status and the psychological stresses of child rearing (155). Thus, well-controlled animal studies are critically important. We used rats that had undergone five pregnancies as our animal model. The objective of our study was to determine the long-term effects of parity on cardiovascular regulation.

Based on the evidence that there are hormonal (12,24) and metabolic (78,98,195) alterations during pregnancies, some of which persist long after reproductive activity has ceased, we hypothesized that multiparity would modulate the vascular response to sympathetic stimulation. Specifically, repeated pregnancy would potentiate pressor response to exogenous administration of the sympathomimetics, and to endogenous sympathetic activation (air jet induced stress). Furthermore, we proposed that such an augmented response would be mediated through increased constriction of the arterial resistance vasculature in the parous animals. To this end we measured mean arterial pressure (MAP) and heart rate (HR) responses to vasoconstrictors and to acute stress in conscious Repeatedly Bred (RB) and Virgin control rats. In addition, we compared reactivity and compliance in isolated small mesenteric arteries.

The venous part of the circulation plays a critical role in cardiovascular regulation (73,197). Given that there are significant alterations in the venous vasculature during pregnancy (92-95), we also tested the hypothesis that these would persist post partum to cause long-term functional and structural changes in the parous animal. Our study aimed to compare venous tone and venous compliance of RB and Virgin rats; to this end we measured mean circulatory filling pressure (MCFP) and reactivity and compliance of isolated small mesenteric veins. We also compared the effects of volume loading on the blood pressure responses of RB and Virgin rats.

The data supported our hypotheses, namely that repeated pregnancy augments the pressor response to intravenous infusion of vasoconstrictors, phenylephrine (PE) and noradrenaline (NA), and to acute stress, and this is due, at least in part, to changes in both the passive and active characteristics of the mesenteric arterial vasculature. The results of our study with inhibition of nitric oxide synthase (NOS) and cyclooxygenase (COX) suggest that both nitric oxide (NO) and prostaglandins contribute to these alterations. Furthermore, repeated pregnancy induces a long-term reduction in splanchnic venous compliance, and augments splanchnic venous reactivity. This compromises the ability of the capacitance (venous) system to accommodate volume overload and to buffer changes in cardiac preload. Thus repeated pregnancy potentiates both afterload and preload response under conditions where there is an activation of the sympathetic nervous system, such as in acute stress. We propose these transient changes in afterload and preload could, in time, contribute to the increased risk for cardiovascular disease observed in multiparous individuals.

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ABBREVIATIONS:

BH4	Tetrahydrobiopterin
CO	Cardiac Output
CVP	Central Venous Pressure
DBP	Diastolic Blood Pressure
eNOS	Endothelium Nitric oxide Synthase
FAD	Flavin Adenine Nucleotide
HEPES-PSS	HEPES-Buffered Phosphate Saline Solution
HR	Heart Rate
I.V.C.	Inferior Vena Cava
LDL	Low Density Lipoprotein
L-NAME	NG-Monomethyl-L-Arginine
MAP	Mean Arterial Pressure
MCFP	Mean Circulatory Filling Pressure
NA	Noradrenaline
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NO	Nitric Oxide
NOS	Nitric Oxide Synthase

PE	Phenylephrine
PGHS	Prostaglandin H Synthase
RAS	Renin-Angiotensin-Aldosterone System
RB	Repeatedly Bred
RIA	Radio Immuno-Assay
SBP	Systolic Blood Pressure
TPR	Total Peripheral Resistance
VSMC	Vascular Smooth Muscle Cells

1 INTRODUCTION

1.1 Overview:

Pregnancy is associated with profound alterations in the cardiovascular system (7,26,200). Cardiovascular parameters such as blood pressure (130,185,214), cardiac output (126,169,186), blood volume (37,100), vascular reactivity (50,64,92) and vascular compliance (30,97) are all modulated during pregnancy. Some, but not all, of these variables return to normal (pre-pregnancy) levels at parturition. However, the time course of resolution of these changes and their long-term consequences on cardiovascular system remains to be elucidated.

Epidemiological evidence suggests that while parity, having borne offspring, provides protection against breast (131), colorectal (51) and ovarian (69) cancer, it also independently and significantly increases the risk of cardiovascular disease (117). It has been reported that parous women have an augmented morbidity and mortality from ischemic/degenerative heart disease (153,155), cerebrovascular disease (153), and hypertension (10). There is also evidence from animal studies that repeated pregnancy adversely affects the cardiovascular system. In rats, it has been reported that repeated pregnancy is associated with degradation of vascular elastic tissue (210) and an increase in the incidence of spontaneous arteriosclerosis of the aorta, and of the mesenteric and renal vascular beds (211,213). It has also been reported that repeated pregnancy attenuates the production of NO in the kidney, which results in increased vasoconstriction in renal blood vessels (168).

Cardiovascular disease is the leading cause of death and disability in women (3). It is thus essential to understand the risk factors and the patho-physiological processes behind the development of cardiovascular disease. In the current research project, using a rat model, we studied the long-term effects of repeated pregnancy on cardiovascular homeostasis, with a view to understanding how it may effect the development of cardiovascular disease later in life.

1.2 Overview Of Circulation: Medical Physics Of Blood Pressure Homeostasis:

The circulatory system, which is responsible for the movement of blood and lymph in the body, comprises a system of blood vessels, lymph vessels and the heart (159). The basic function of the circulatory system is to service the requirements of the tissue and to maintain homeostasis. This includes transport of nutrients, oxygen, hormones etc. to the tissues, removal of waste products, and control of thermo-regulation. In order for it to carry the above mentioned functions, it is pivotal for the circulatory system to maintain a constant range of perfusion pressure. Blood pressure control can be classified into long- and short-term control. Long-term control of blood pressure is achieved by regulation of total body fluid and salt by the kidney, however for the purpose of this thesis I will focus on the short term control of blood pressure.

Short-term homeostasis of blood pressure is a complex process which is maintained through the interaction of several regulatory mechanisms. These mechanisms maintain blood pressure by modulating cardiac output and total peripheral resistance (TPR). As shown in Figure 1, both the arterial and the venous half of the circulatory systems play important roles in maintaining TPR and cardiac output.

1.2.1 Contribution of arterial system in blood pressure regulation: In part, the arterial system maintains blood pressure by modulating the TPR (Figure 1).

The TPR is the impediment to blood flow in the circulatory system (157). Mathematically, TPR or resistance (R) is represented by Darcy's Law: the ratio of cardiac output (Q) to the pressure gradient (ΔP) between the arterial system and the venous system (4).

$$R = \frac{\Delta P}{Q} \qquad (1)$$

Since the biggest drop in pressure occurs across the arterioles, TPR primarily depends upon arterial tone, specifically on arteriolar diameter (38). Under normal physiological conditions, the flow in blood vessels is laminar. This means that the blood flows in the form of parallel sheets (laminae). Therefore the lamina directly in contact with the vessel wall has the maximum resistance to flow and its flow velocity is zero. In vessels with a smaller diameter, most of the blood is in contact with the vessel wall and therefore the rapid flowing central laminae do not exist. Thus, the smaller the vessel, the greater the resistance, and slower the flow (118). This relationship of radius and resistance can be written as Poiseuille's Law, which states that the R to flow of a fluid is directly proportional to the fluid viscosity (η) and the length of the vessel (L), and inversely proportional to the fourth power of the radius (r) of the vessel.

$$R = \frac{8\eta L}{\pi r^4} \qquad (2)$$

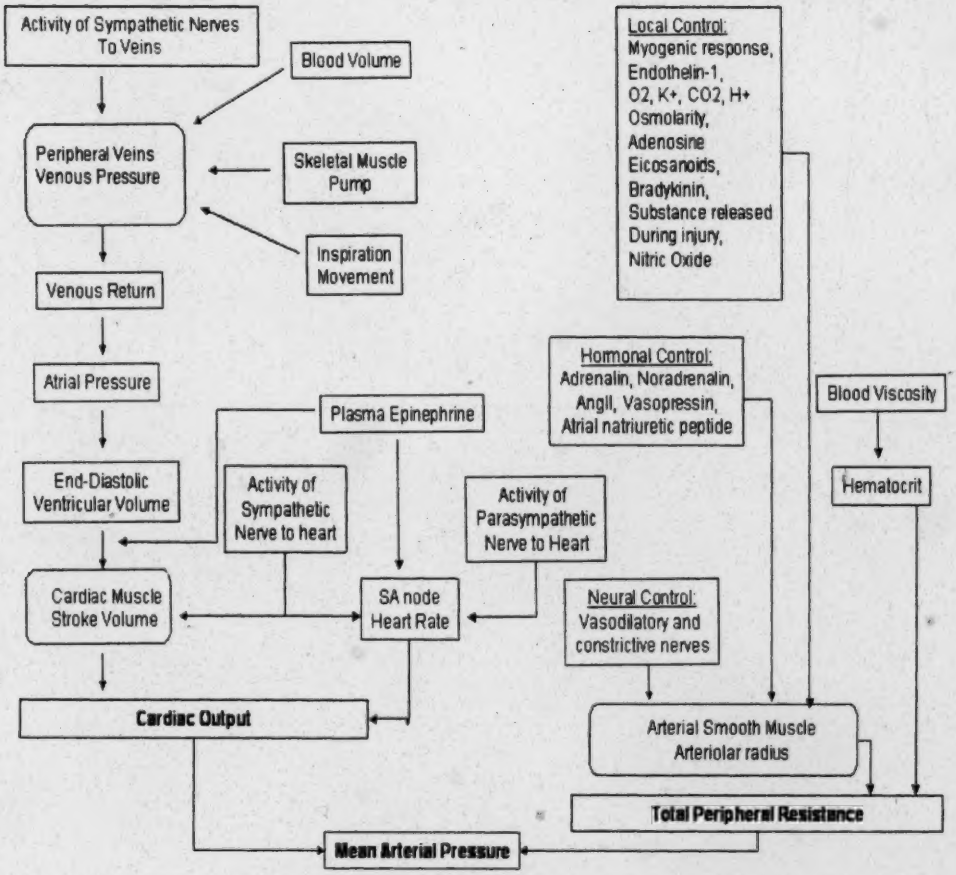

Figure 1: Summary of Factors that determine systemic arterial pressure via venous and arterial system. Adapted from (215). O_2: oxygen, K^+: potassium ions; Co_2: carbon dioxide; H^+: proton.

Combining Darcy's law and Poiseuille's law (equation 1 and 2):

$$\Delta P = Q \frac{8\eta L}{\pi r^4} \qquad (3)$$

From equation 3, it can be seen that even a small change in the caliber of the vessel will have a great effect on the TPR and thus on the arterial blood pressure.

Arterial diameter is actively controlled by the vascular smooth muscle cells (VSMC) present in its tunica media (middle layer of the arterial wall). Increased tone of smooth muscle cells decreases the diameter of the blood vessel and thus increases the resistance to flow and vice versa. There is always some degree of basal tone (intrinsic tone) in the smooth muscle cells (178), which can be modulated by factors which may be broadly classified as the intrinsic or extrinsic regulatory factors (Figure 2) (204).

One of the primary intrinsic modulators of VSMC tone is the endothelium lining of the blood vessels (75,102,201). Endothelium cells secret several vasoactive substances which can diffuse to the adjacent smooth muscle cell layer to alter its contractility. These substances can be broadly classified as endothelium derived relaxing factors and endothelium derived contracting factors (203). However, for this thesis I will focus on the endothelium derived relaxing factors, specifically, NO and prostaglandins.

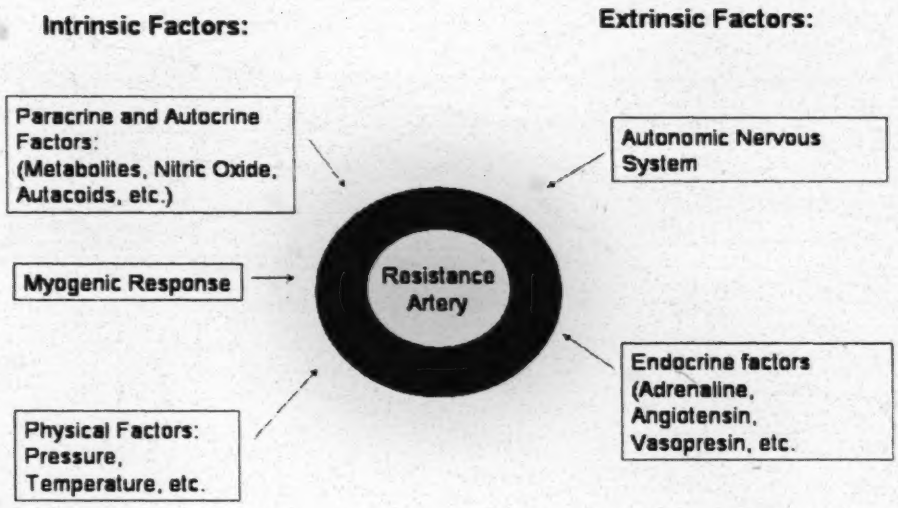

Figure 2: Overview of intrinsic and extrinsic factors that control vascular tone in resistance arteries. Adapted from (119).

One of the first endothelium-derived mediators to be discovered was prostacyclin, a type of prostaglandin (eicosanoids) (23). Prostacyclin is a potent vasodilator which is produced from arachidonic acid by the action of prostaglandin H synthase (PGHS) and prostacyclin synthase (Figure 3) (48). Arachidonic acid also produces other prostaglandins such as thromboxane and its precursor PGH_2. Both thromboxane and PGH_2 are potent vasoconstrictive agents (48). Under normal physiological conditions the prostaglandins produced are predominately vasodilators such as prostacyclin. However, in vascular pathologies such as hypertension and diabetes, thromboxane and PGH_2 are the major mediators produced (48). Interestingly, all these conditions are associated with increased oxidative stress (76,77). Furthermore, oxidative stress has been shown to potentiate PGHS-2-dependent vasoconstriction (49,190) thereby suggesting that an increase in the production of free radical may be a major link between vascular disease and increased prostaglandin-dependent vasoconstriction.

Another endothelium-dependent vasodilator was discovered in 1980 when Zadwadski and Furchgott observed that the arterial vasodilatory response to the acetylcholine was changed to vasoconstriction when the vessels were denuded of endothelium (63). It was discovered that acetylcholine stimulates endothelium cells to secrete an endothelium-derived relaxing factor, which diffuses to VSMC to cause vasodilatation. In 1987, this endothelium-derived relaxing factor was identified as NO (143,164). NO is a highly volatile molecule synthesized by both endothelium cells (9) and VSMC (32), from L-arginine by the action of nitric

oxide synthase (NOS). NOS exists in three isoforms: neural NOS (nNOS or NOS I), endothelium constitutive NOS (eNOS, ecNOS or NOS II) and inducible NOS (iNOS or NOS III) (112,142). Both nNOS and eNOS are calcium-dependent enzymes, thus producing NO in a controlled fashion. Inducible NOS is calcium independent isoforms of NOS and its activation results in an uncontrolled production of NO. As shown in Figure 4, NO mediates vasodilatation primarily by its direct action on guanylate cyclase and subsequent production of cyclic guanosin monophosphate (29). In addition, NO also reduces vascular tone by inhibition of sympathetic nerve induced vasoconstriction (224).

It is important to note that, under normal physiological conditions, NOS produces NO. For this reaction to occur, NOS requires co-factors such as coenzyme nicotinamide adenine dinucleotide phosphate (NADPH), Flavin adenine nucleotide (FAD), oxygen, tetrahydrobiopterin (BH_4), heme and calmodulin (25). However, in various pathological conditions such as hypertension there may be a reduction of these cofactors (BH_4) which results in uncoupling of NOS. This switches NOS-mediated production from NO to superoxide anion (116).

Thus NO plays a key role in the regulation vascular tone in both physiological and pathological conditions.

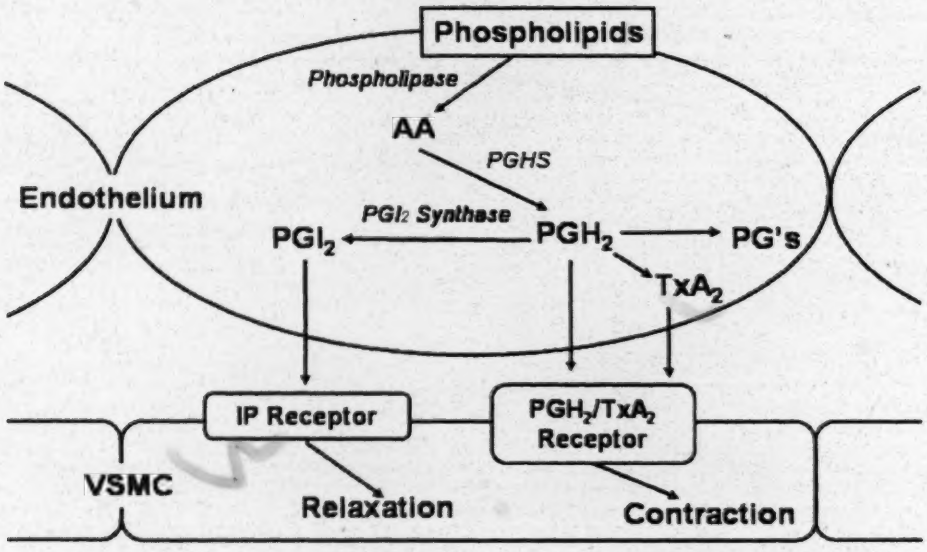

Figure 3: Simplified schematic of the synthesis and effects of prostaglandins on vascular smooth muscle cells (VSMC). Adapted from (48). AA: arachidonic acid, PGHS: prostaglandin H synthase, PGI_2: prostacyclin, TxA_2: thromboxane, PG's: other prostaglandins

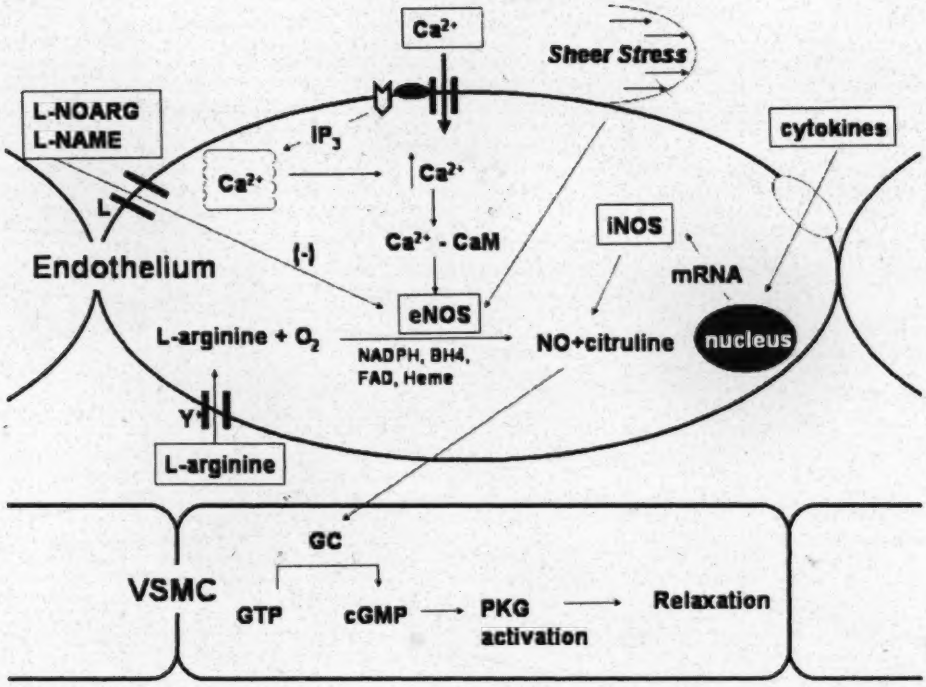

Figure 4: Simplified schematic of the mechanism of NO production in the endothelium cells and NO induced vasorelaxation of vascular smooth muscle cells (VSMC) Adapted from (40). IP_3: inositol triphosphate, eNOS: endothelium/constitutive nitric oxide synthase, iNOS: inducible nitric oxide synthase, NADPH: nicotinamide adenine dinucleotide phosphate, FAD: flavin adenine nucleotide, BH4: tetrahydrobiopterin, CaM: calmodulin, GC: guanylate cyclase, GTP: guanosine triphosphate, cGMP: cyclic guanosine monophosphate, PKG: protein kinase G.

1.2.2 Contribution of venous system in blood pressure regulation: The venous system was long believed to be a mere network of conduit vessels through which blood from the capillaries returns to the heart. However, it is now well established that the role of the venous system in cardiovascular regulation extends far beyond this simplistic view (197,199). The venous system is complex and has a multifunctional role. It acts as a reservoir for the total blood volume, as a selective barrier between the intra- and extra-vascular spaces, and as the regulator of cardiac filling pressure (144). It also plays a role in angiogenesis, defines postcapillary resistance, and synthesizes several biologically active substances (144).

The venous system is the primary reservoir for the blood (172). To fulfill the criteria of a blood reservoir a vascular bed must possess two qualities: it must be capable of containing a significant amount of blood volume, and it must be capable of rapidly buffering changes in total blood volume in a controlled and defined manner (74). The venous system contains approximately 70% of total blood volume, and is under direct control of the sympathetic nervous system (172). Change in venous tone can increase pooling or mobilize a significant volume of blood when the total blood volume is increased or decreased respectively; this helps to maintain a constant range of cardiac output and blood pressure (197,199).

Venous capacity is defined as the volume of blood contained in the venous

system at a given distending pressure, whereas venous capacitance is the relationship between contained volume and the distending pressure (166). The total blood volume contained in the vascular system can be classified into unstressed and stressed volume (166). If we think of the vascular system as a set of interconnected elastic tubes, then the volume of blood required to fill this system just enough so that there is no increase in the intraluminal pressure is called the unstressed volume (165). Therefore, unstressed volume is also known as the hemodynamically inactive part of the total blood volume as it doesn't cause any change in cardiovascular parameters. However, if a greater volume of blood is pumped into this system such that it starts to distend the elastic tubing, this will cause an increase in intraluminal pressure. Therefore, this "additional" volume of blood which causes the increase in intraluminal pressure is called the stressed volume (165). Stressed volume is the hemodynamically active part of the total blood volume.

A decrease in total blood volume, such as occurs in hemorrhage, through activation of baroreflex system, induces a reflex increase in sympathetic outflow to the venous circulation, which causes an increase in venous tone (80). The increased venous tone then decreases venous capacity, thus decreasing the unstressed volume. Taking into account the law of conservation of mass, the decreased unstressed volume must then be converted into stressed volume. This stressed volume is then mobilized towards the heart thus increasing venous return to the heart and cardiac preload (173). According to the Frank-Starling law,

cardiac preload is the major determinant of stroke volume (199). Cardiac output is the product of HR and stroke volume. Thus an increase in venous tone will, through an increase in venous return, increase cardiac output and buffer the hemorrhage-induced fall in blood pressure.

The ability of the vascular system to contain a given volume of blood also depends upon its compliance (73,165). Compliance can be defined as the ratio of change in pressure to change in volume ($C=\Delta V/\Delta P$) (73). If a vessel is more compliant it is able to accommodate a greater volume of blood without changing pressure than a vessel which is less compliant. Compliance can be mathematically calculated by measuring the slope of the Volume: Pressure relationship (181). Veins are highly compliant and are therefore the primary reservoir of the total blood volume (73,166). Venous compliance depends upon the elastic properties of the vessel. Changes in venous tone change the vascular mechanical properties and change venous compliance. Thus, in acute situations, an increase in venous tone (165) either by activation of sympathetic system or by hormonal factors, can decrease venous compliance (73,182).

Venous tone is under direct control of the autonomic system as well as hormonal and local factors. Veins are primarily innervated by the sympathetic arm of the autonomic nervous system (166). Stimulation of sympathetic nerves can cause a reduction in venous volume by 60-70 % (156). In her review, Pang reported that the primary subtype of adrenergic receptor responsible for venoconstriction is species-dependent (166). In *in-vivo* studies, α-1

adrenomimetics increased venous tone in dogs but not in rats (167). On the other hand, Galligan JJ *et al.* reported a significant constriction in isolated rat mesenteric veins to PE (128). It is possible that the technique used and the type of venous bed studied, rather than the species difference, are responsible for the different venous adrenergic response observed in these two studies.

The splanchnic vascular bed, which comprises the blood vessels of liver, spleen, and gastrointestinal tract, holds about 33% of the total blood volume. It is thus the most important determinant of total vascular capacity (72). Greenway *et al.* reported that in cats, 65% of the blood volume removed from or infused into the animals may be derived from, or pooled in, the splanchnic vascular bed (74). Using blood pool-scintigraphy, Scott-Douglas *et al.* showed that in dogs, modulation of intestinal capacity in the range of approximately 95% to 135% of initial venous capacity, was able to maintain cardiac output constant during volume loading or hemorrhage respectively (179). Thus a sigmoid buffer-action curve is observed by which change in venous capacity buffer changes in cardiac output during acute volume loading or hemorrhage (179,197). Furthermore, Shoukas and Hasse showed that in rats, bilateral occlusion of the carotid sinus causes a simultaneous decrease in the diameter of the splanchnic veins; this vascular bed was later shown to account for 80% of the total volume reflexly displaced from the microcirculation during hypotension caused by bilateral carotid occlusion (80,81).

As in arteries, changes in venous tone caused by constriction/relaxation of smooth muscle cells can also be modulated by the venous endothelium. Acetylcholine, an endothelium-dependent relaxing agent, causes a transient relaxation in isolated canine femoral, pulmonary and splenic veins (53). In cat skeletal muscle veins, the specific NOS inhibitor NG-monomethyl-L-arginine (L-NAME) causes a 23% constriction which may be reversed with L-arginine (59). The venous endothelium also contains endothelin receptors and activation of these receptors, specifically the endothelin-B receptors, causes release of NO (104). In conscious rats, Glick *et al.* reported that L-NAME causes a dose-dependent increase in whole animal total venous tone, which could be reversed by L-arginine infusion (68). However, Wang *et al.* failed to see any such change (208), although they did find an increase in venous resistance.

As in arteries, the venous wall consists of three layers. The innermost layer which lines the lumen consists of endothelium cells attached to the basement membrane; it is called the Tunica Intima. The middle layer, primarily consisting of smooth muscle cells, is called the Tunica Media. The outermost layer consists of connective tissue, and is called the Adventitia. Compared to the arteries of the same order, veins contain approximately ten times less smooth muscle cells, which make the venous wall significantly thinner. Collagen is the primary connective tissue component responsible for the mechanical characteristics (compliance) of the veins.

1.3 Characterization Of Vascular Reactivity:

One of the major pathophysiological mechanisms underlying cardiovascular disease is the alterations in reactivity of resistance sized blood vessels. Vascular reactivity can be studied at different levels of organization, from intact animal down to isolated blood vessels. In intact animals, blood vessels are under the influence of extrinsic factors such as nervous and endocrine system. Studying in-vivo vascular reactivity is critical to understanding the physiological function of blood vessels in an integrated system. However, that same influence of extrinsic factors makes interpretation of vascular reactivity responses complex. *In-vitro* techniques, on the other hand, provide a means to study vascular reactivity under relatively controlled conditions, and may provide information regarding the intrinsic characteristics of a particular vascular bed.

1.3.1 *In-vivo* techniques: The results of studies of vascular reactivity depend upon: 1. vessel type: artery or vein, 2. response measured: MAP, resistance, conductance or mean circulatory pressure, and 3. drug delivery route: whole body infusion or local infusion.

Whole animal arterial reactivity can be measured by infusing a vasoconstrictor and measuring the MAP response (Figure 5). The infused vasoconstrictor increases resistance at the level of the arterioles (Area A), which results in an increase in the upstream pressure measured by a pressure transducer (PTA in Figure 5). Thus the greater is the reactivity of the arterioles; the greater

is the rise in pressure. However, as the blood pressure also depends upon cardiac output, it is important that the cardiac output remain constant. Thus it is necessary to be aware of any cardiac effects that vasoconstrictor may have. This technique is also limited in that it measures *whole body* arterial reactivity. To overcome this problem, two approaches can be used to study the vascular function of a particular vascular bed *in-vivo*: 1. intra-vital microscopy and 2. localized infusion of vasoactive agent. In the first technique, blood vessels, usually from the mesenteric or cremaster vascular bed, are exteriorized and are visualised using light microscope. Vascular reactivity is then measured in terms of changes in diameter in response to a vasoactive agent. In second technique, the vasoactive agents are locally infused through a very fine cannula implanted into a specific vascular bed, such as that of hind limb, renal or mesentery. Responses are then measured in terms of changes in local blood flow. Intra-vital experiments are conduced on anesthetised animals. However experiments using local infusion of drug can be performed on conscious animals.

In-vivo estimation of venous tone requires a different approach. Unlike the arterial system, resistance in the distal end of the venous system (terminal resistance at Area V in Figure 5) is almost negligible. Therefore, although infusion of a vasoconstrictor would cause an increase in overall venous tone, this would not be reflected by an increase in venous pressure as measured by the pressure transducer (PTV in Figure 5). This is because the lack of terminal resistance at Point V would allow blood from the venous side to be simply

mobilized towards the heart without a significant change in the pressure measured by PTV. To overcome this problem, Guyton suggested measuring the equilibrium pressure or the MCFP (mean circulatory filling pressure) (79). MCFP, an index of venous tone, is defined as the equilibrium pressure in the circulatory system when the blood flow is stopped in that system. MCFP primarily depends upon the total blood volume in the system and the state of tone or compliance of that system. Venous compliance is approximately 60 times that of the arterial compliance and therefore MCFP is primarily dependent upon venous tone (166). Therefore, if blood volume is kept constant, an increase in MCFP would denote a decrease in venous compliance or increase in venous tone, and vice versa.

1.3.2 ***In-vitro*** **techniques:** *In-vitro* techniques to study vascular reactivity depend upon the size of the vessel and on the response measured. Two different techniques that are available are wire myography and pressure myography. Wire myography measures changes in tension (146) whereas pressure myography measures changes in diameter (82), as indices of vascular reactivity. In wire myography, an isolated vessel is threaded on two metal wires, where one wire is connected to the fixed arm of the myograph and the other wire is connected to a force transducer. The vessel is stretched on these two wires such that changes in the tone of the vessel can be measured by the force transducer. In pressure myography, the vessel is cannulated with two glass cannulae. The vessel is then set at a physiological intraluminal pressure and vascular reactivity responses are measured as changes in diameter. Pressure myography can be customized to

study the effect of flow on vascular reactivity by exposing the cannulated vessel to intraluminal flow. In the wire myograph system, the vessel is threaded on two wires. It thus becomes technically difficult to use this system to study very small arterioles (<100μm). However, these smaller vessels can be cannulated and their reactivity can be measured using pressure myography.

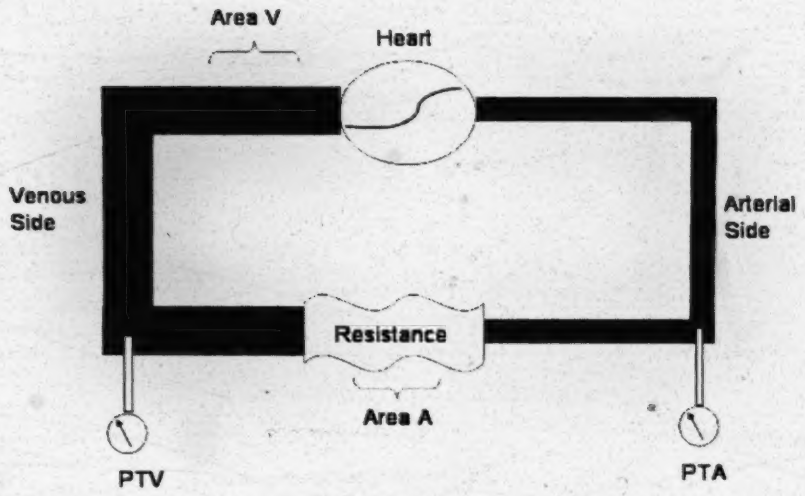

Figure 5: Simplified schematic of the circulatory system. Area A: pre-capillary resistance, Area V: terminal venous resistance, PTA: pressure transducer implanted in aorta, PTV: pressure transducer implanted in vena cava.

1.4 Cardiovascular Adaptation During Pregnancy:

In order to provide optimum in-uterus environment to the growing fetus, nearly every aspect of the circulatory system in the maternal body undergoes some degree of adaptation during pregnancy. One of the most significant changes is a 50% increase in the total blood volume. In humans, stroke volume increases by 75% and HR by 15 beats/min (157). This in turn, significantly increases cardiac output during pregnancy. Although total blood volume and cardiac output are increased during pregnancy, MAP falls due, primarily, to a decrease in TPR (171).

1.4.1 Arterial system: During pregnancy, TPR decreases dramatically. Reduction in TPR induces a state of relative underfilling of the arterial side of the circulation, which activates the renin-angiotensin-aldosterone (RAS) system. Activation of RAS system increases plasma level of both renin and angiotensin which, in humans, continue to rise until the 30th week of pregnancy (27). Although plasma levels of renin and angiotensin are increased during pregnancy, vascular reactivity to angiotensin is significantly blunted (1). Attenuated responses to angiotensin and to other vasoconstrictors such as epinephrine are also observed in species other than humans, including rabbits (13) and rats (163).

The low resistance uterine circulation which develops during pregnancy contributes to the reduced TPR. However, the decrease in TPR begins to occur very early in pregnancy, even before the uterine circulation becomes

physiologically important (194). This suggests that there are other mechanisms which also play a role in the reduction of TPR.

Several mediators, local and circulating, have been proposed to be responsible for this attenuated vasoconstriction response to pressor agents. During pregnancy, synthesis of prostacyclin is increased in several species including humans, sheep and rats. Prostacyclin is a potent vasodilator which is produced from arachidonic acid by the action of prostaglandin H synthase and prostacyclin synthase (48). Thus, inhibition of prostaglandin H synthase augments vasoconstriction to catecholamine and angiotensin II (22) and attenuates vasorelaxation to endothelium-dependent vasodilators such as methacholine (44,45). However, inhibition of prostaglandin system does not completely reverse the altered vascular response observed during pregnancy. This suggests that prostaglandins are responsible, only in part, for modulation of vascular reactivity during pregnancy.

During pregnancy, there is also a significant increase in the production of NO (132,184). Mesenteric and uterine arteries from pregnant rats have an enhanced sensitivity to the endothelium-dependent vasodilator acetylcholine compared to non-pregnant rats (39,66,110,151). Inhibition of NOS significantly reduces this increased sensitivity (150). Inhibition of NOS also prevents the attenuated PE reactivity of isolated mesenteric arteries from pregnant rats (227). Furthermore, the *in-vivo* pressor responses to vasoconstrictors such as angiotensin, NA, are potentiated by infusion of the NOS inhibitor L-NAME

such that the pressor response between the late-pregnant rats and non-pregnant rats are equalized (141). This suggests that NO plays a significant role in cardiovascular adaptation during pregnancy.

1.4.2 Venous system: In order to maintain an increased cardiac output throughout pregnancy, the venous system also undergoes significant changes. Both venous compliance and reactivity to vasoconstrictors are modulated during pregnancy. The compliance of limb and uterine veins increases during pregnancy (187), whereas mesenteric venous compliance decreases (93-95). The increase in uterine venous compliance is associated with an increase in the elastin content, whereas the pregnancy-induced decrease in mesenteric venous compliance is associated with a decrease in collagen content (93,162). The differential effect of pregnancy on different vascular beds reflects their very different functional roles; the splanchnic veins are important not only as conduit vessels, but also as regulators of blood distribution and cardiac preload (73,80,161), both of which are known to increase during pregnancy. Studies to determine whole body compliance have yielded conflicting results. There has been evidence for compliance being increased (52), decreased (99), or not changed (30) during pregnancy. Although Humphreys & Joels reported a pregnancy-induced increase in MCFP which was independent of sympathetic tone, they found total body compliance to be increased (97). Part of the difficulty in evaluating these results arises from the fact that both the splanchnic and peripheral vascular beds contribute to whole body compliance, and that the degree to which each

contributes probably varies according to the experimental conditions (anesthesia, species etc).

Despite the fact that venous reactivity of the splanchnic vascular bed to exogenous norepinephrine increases during pregnancy, the venous response to transmural nerve stimulation declines (92). Hohmann *et al.* attributed this difference in venous responses to alterations in catecholamine reuptake mechanism during pregnancy (92). It has also been reported that the pregnancy-induced increase in exogenous norepinephrine sensitivity is highly dependent on vascular intraluminal pressure, and that increases in intraluminal pressure potentiate norepinephrine sensitivity in mesenteric veins (95).

1.5 Parity As A Risk Factor For Cardiovascular Disease In Women:

There have been several human epidemiological studies on the association between reproductive history and subsequent cardiovascular mortality/morbidity in women, and most have found that repeated pregnancy is associated with a significant increase in the risk of development of cardiovascular disease. Beral conducted one of the first studies to examine the cause of death in > 1.2 million women in England and Wales between 1938 and 1960. She reported that there was a 20% increase in the death from cardiovascular disease (hypertension, ischaemic and degenerative heart disease and cerebrovascular disease) in multiparous compared to nulliparous women aged 45-74 years (10,71).

Following this report, there were numerous other studies conducted to look at the relationship between parity and risk of cardiovascular disease in different population groups (18,41,46,98,117,152-154,188,191,192,217). In the Framingham Heart Study and in National Health and Nutrition Examination Survey National Epidemiologic Follow-up Study, a cohort of 2357 and 2533 women were followed for 28 and 12 years respectively. It was found that there was a greater increase in the coronary heart disease among multigravid women than among women who had never been pregnant, a difference which reached statistically significant in women with more than five pregnancies (153).

Although, there is a strong association between the number of pregnancy and the risk of developing cardiovascular disease, this association has probably been underestimated due the following reasons (117). Most of the above mentioned studies had survival bias, where women with greater number of pregnancies were likely to die earlier due to cardiovascular disease and thus increasing the number of cardiovascular disease in women with lower number of pregnancies. Furthermore, conditions such as polycyctic ovary syndrome and preeclampsia, which are related to both infertility and an increase in cardiovascular disease, may result in an increase in cardiovascular disease in women with low number of pregnancies.

It has also been reported that, relative to nulliparous women, parous women have an overall 36% greater risk of carotid atherosclerosis, which rises to 64% in women with >3 children (98). A more recent meta-analysis conducted by Lawlor *et al.* reported a significant increase in risk of cardiovascular disease in women with more than 2 pregnancies (117). Population in this study was a more homogenous subset of population; women were post-menopausal with an average age of 69-80 years. Interestingly, they also reported a higher incidence of cardiovascular disease in men with multiple children. Development of cardiovascular disease involves an interaction of complex, yet to be fully described, patho-physiological processes and it has become increasingly evident that these processes may be modulated by confounding factors (188). Indeed, there are several confounding factors associated with childbearing that have

been shown to influence the risk of cardiovascular disease (6), including body mass index, exercise, smoking, alcohol, stress level associated with raising a big family, social class, education level and employment history. Interestingly, standardizing the results of the above mentioned studies with the known confounding factors attenuated the associations between greater number of children and cardiovascular disease in both men and women; although in women some association remained (117). This suggests that there is/are independent biological factor(s) responsible for an increase in the cardiovascular disease in multiparous women.

1.6 Effects Of Parity On Cardiovascular System:

Although there is ample evidence to suggest that repeated pregnancy is a risk factor for cardiovascular disease, there have been very few systemic animal studies conducted to investigate this association. Wexler *et al.* reported that there is a significant increase in the incidence of arteriosclerosis in RB rats (211); RB female rats develop grossly visible arterial plaques in the aorta, coronary, renal, mesenteric and cerebral arteries. It has also been reported that repeated pregnancies causes degradation of elastin fibers in the medial layer of these arteries. This was also associated with an increase uptake of ^{45}Ca in the arterial wall of RB (105). Wexler *et al.* also found that RB female rats develop hyperglycemia, hyperlipidemia, hyperuricemia and hypertension (137,209). These authors suggested that the increase in systemic blood pressure was linked to modulation of mineralocorticoid activity during pregnancy and lactation (137).

The kidneys play a key role in long-term blood pressure regulation. There is evidence from animal studies that pregnancy has long-term effects on renal function. It has been reported that RB rats have an increased kidney weight (121,137). It has also been reported that renal arteries from aged RB rats have significant greater tone than those from control Virgin rats (168). This increase in renal vasoconstriction is associated with attenuated NO production in the renal vessels. Interestingly, L-name also caused a smaller decrease in MAP in RB than Virgin rats, suggestion that there is less influence of NO in maintaining basal

MAP in RB than in virgin rats. By contrast, Baylis and Rennke did not find any structural or functional difference between the kidneys from RB and Virgin rats (8), an inconsistency which Reckelhoff attributed to a specie-dependent difference (168).

1.7 Effects Of Parity On Endocrine System:

Pregnancy is a state of profound endocrine alterations in the maternal body. Given the importance of the influence of endocrine factors on the cardiovascular system, it is important to consider the long-term effect of repeated pregnancy on the hormonal system. Indeed, the effects of parity on the endocrine system have been extensively studied with respect to conditions such as breast, ovarian and colon cancer. It has been reported that parous women have decreased plasma levels of estrogen compared to nulliparous women, which has been attributed to shorter menstrual cycles (12). There is also evidence for a reduction in estrogen receptors in the mammary gland tissue of RB rats (193,221). Other studies have shown that estrone, dehydroepiandrosterone sulphate and dehydroepiandrosterone levels are lower, and estriol levels higher in multiparous compared to nulliparous women (42,56,109,148). It is important to note that the above-mentioned endocrine changes were observed in pre-menopausal women and that the differences in these reproductive hormones were abolished when they reached menopause (28,152). During pregnancy there is a significant increase in plasma levels of total cortisol and transcortin (corticosteroid binding globulin; CBG), as well as unbound cortisol, as compared to non-pregnant women (2). The alteration in cortisol levels during pregnancy has also been reported in other species (14,108) including rats (222). Interestingly in RB rats, the medullary cells of the adrenal gland were reported to be hyperplastic and pleomorphic, indicating adrenal

overactivity (212). This suggests that increased activity of adrenal system may persist long after reproductive activity has ceased.

Parity has also been shown to modulate the endogenous opioid system. Multiparous rats are less sensitive to the effects of morphine on maternal behavior than are primiparous rats (134,135). Furthermore, reproductive events, including pregnancy and lactation, have been shown to increase opiate receptor density (20). Specifically, it was found that opiate receptor density in the medial preoptic area is significantly greater in multiparous than in primiparous rats (83). The endogenous opioid system also plays a key role in the stress response. Therefore parity-induced alterations in this system may influence the physiological response to stress in parous rats. It has also been reported that morphine lowers both baseline and stimulated plasma prolactin levels in parous animals, possibly through its inhibitory action on dopamine secretion (19,24,149). This is of significance given that prolactin has a bimodal effect on the cardiovascular system; high plasma levels of prolactin attenuate and lower levels augment the reactivity of isolated aortic strips and mesenteric vessels to NA and angiotensin II (133,147). *In-vivo*, intravenous infusion of prolactin has a similar bimodal effect on blood pressure (139). We suggest that the cardiovascular differences observed between parous and nulliparous rats may thus be attributed, in part, to the reduced plasma levels of prolactin.

1.8 Effects Of Parity On Metabolic And Oxidative State:

Pregnancy is a state of marked metabolic alterations (78). Many studies have shown there to be a several-fold increase in the serum levels of total cholesterol, low density lipoprotein (LDL) including the small dense LDL subfraction, and triglyceride levels, as well as a reduction in the levels of high density lipoprotein (78,117,216). This suggests that a significant atherogenic profile persists during pregnancy (136). It has also been reported that, during pregnancy, the body is under increased oxidative stress (60,103,176), and that bio-markers of oxidative stress in plasma are significantly elevated (195). This pregnancy-induced atherogenic lipid profile persists for several years post-partum, long after reproductive activity has ceased. (78,98,117). LDL and specifically the small dense LDL subfraction are highly susceptible to oxidation, which can cause endothelium damage through several pathways (158). Oxidized LDL reduces the bioavailability of vasoprotective NO (33,87). Furthermore, LDL increases oxidative stress in blood vessels either directly by increasing the production of reactive oxygen species such as superoxide ion and oxidized lipid, or by recruiting macrophages to generate superoxide ions, which can further decrease the bioavailability of NO (43,158,206).

1.9 Experiment Model:

It is difficult in epidemiological studies to control for potentially confounding factors such as socio-economic status and the psychological stresses of child rearing (155). Thus, well-controlled animal studies are critically important. In the present study we used the rat as our experimental model; the pregnancy-associated changes observed in rats are very similar to those found in humans (183). Furthermore, in rats, we can induce repeated pregnancies within a short interval of time. Seven to eight month-old female Long Evans (specific pathogen-free) RB rats were obtained from Charles River, St Foy, Quebec, Canada; these animals had undergone five pregnancies. The control animals were aged-matched Virgin rats, also from Charles River, which had been raised in the same living conditions as the RB rats.

1.10 Experiment Preparations And Hypothesis:

We utilized the following experimental preparations during our investigations:

1. *In-vivo* experiments using conscious unrestrained rat implanted with: *1)* inferior vena cava (i.v.c.) non-occlusive cannula for drug infusion, 2) Pressure transmitter (PA-C40, Data Sciences International) in abdominal aorta for blood pressure and HR recording and 3) a jugular venous cannula for infusion of Evans Blue dye. Blood pressure and HR were measured during air jet induced acute stress, i.v. infusion of PE, and volume loading.
2. *In-vitro* small vessel wire and pressure myograph systems were used to study the reactivity and compliance of mesenteric and femoral arteries, and mesenteric and saphenous veins.
3. *In-vivo* experiments using conscious unrestrained rats implanted with: 1) right atrium balloon tipped catheter to induce circulatory arrest, 2) two femoral vein cannula for drug infusion and measuring CVP and 3) femoral arterial cannula for measuring blood pressure and HR. CVP and MCFP were measured during infusion of NA.

The first series of *in-vivo* experiments examined the differences in the blood pressure and HR responses to acute stress as well as to PE infusion in RB and Virgin control rats. Based on the evidence that there are vascular, hormonal

and metabolic alterations during pregnancies, some of which persist long after reproductive activity has ceased, we hypothesis:

That parity would modulate pressor responses to acute stress and i.v. infusion of PE

We found that there was indeed a potentiated pressor response to acute stress and to PE infusion. Given that the small resistance sized arteries are the primary site controlling blood pressure responses, we proposed:

That parity would potentiate PE sensitivity in resistance sized mesenteric and femoral arteries.

During pregnancy, arterial compliance is generally believed to increase (58,187) and arterial tone is reduced. However, during pregnancy, compliance of the mesenteric venous circulation is reduced, and tone is potentiated (93,95,96). It has also been reported that there is a reduction in the elasticity of the blood vessels of parous rats (211). Therefore we proposed:

That the compliance of mesenteric vascular bed of RB would be reduced and venous reactivity potentiated compared to that of Virgin control rats.

The splanchnic vascular bed is the most important part of the circulation in

defining total capacitance of the cardiovascular system (174,198). Given that parity causes a reduction in the compliance and a potentiation of PE sensitivity of the splanchnic vasculature, we proposed:

That there would be a greater increase in MCFP, a greater pressor response to volume loading, and a lower plasma volume, in RB rats compared to Virgin control rats.

Given that parous women have lower plasma levels of estrogen as compared to Virgins (12), and that estrogen causes a reduction in the constriction responses of resistance sized arteries accentuation of vascular distensibility (31,120,138), we proposed:

That plasma levels of estrogen would be reduced in RB compared to Virgin control rats.

2 METHODS

The experimental procedures were approved by the local Animal Welfare Committee in accordance with the guidelines issued by the Canada Council on Animal Care. At the completion of whole animal experiments, the animals were euthanized with a lethal dose of Euthanyl (0.3 ml; MTC Pharmaceuticals, Cambridge, Ontario, Canada) injected intravenously.

2.1 Animal And Housing: Seven to eight month-old female Long Evans (specific pathogen-free) RB rats were obtained from Charles River, St Foy, Quebec, Canada. These animals had undergone five pregnancies, their age at first pregnancy being 56 days. They had been mated after weaning at 3 weeks postpartum. The control animals were aged-matched Virgin rats, also from Charles River, which had been raised in the same living conditions as the RB rats. After receiving the rats from Charles River, they were held in the University of Alberta animal facility on a 12-h–12-h light dark cycle, in a humidity and temperature-controlled environment, and allowed access to water and a 0.3% sodium diet ad libitum. A period of at least 1 month was allowed to elapse before they were used in the study.

2.2 Effects Of Parity On The Arterial System:

2.2.1 Effects of parity on pressor response to PE and acute stress:

General surgical procedures: Rats were anesthetized with pentobarbital sodium (62 mg/kg body wt. i.p.), followed by atropine (0.1 ml, 0.4 mg/ ml s.c.). Buprenorphine (0.01 mg/kg s.c.) was given after the completion of surgery. During surgery, the rats were placed on Deltaphase isothermic heating pad (Braintree Scientific, Inc., Mass., USA) to maintain body temperature at ~37°C. Animals were allowed to recover for one week from surgery and to regain their preoperative body weight.

Inferior vena cava (i.v.c.) non-occlusive cannulations for drug infusion: The inferior vena cava was exposed through a midline abdominal incision. Using retracting sutures, blood flow was stopped in the i.v.c. and a tiny hole was punctured using a 21G needle. A cannula (Silastic, ID: 0.51-mm, OD: 0.94-mm) was pushed through the hole into the vein, and advanced towards the heart until its tip lay at the level of the xiphisternum, at which point blood could be aspirated. The cannula was secured with a series of silk (4-0) sutures tied to the psoas muscle and then tunneled subcutaneously to the back of the neck where it was connected to the metal tubing of a pedestal. The pedestal was then sutured to the skin and the wound was closed.

Implantation of pressure transmitter (PA-C40, Data Sciences International) to record blood pressure in conscious rats: The aorta was exposed through a midline abdominal incision. Using retracting sutures, blood flow was stopped in the aorta (for not more than 60sec at one time) and a tiny hole was punctured using a 25G needle, just above the bifurcation of aorta. The tip of the cannula of the pressure transmitter (PA-C40, Data Sciences International) was pushed into the hole and advanced towards the heart such that ~1cm of the tip lay inside the vessel. The tip of the transmitter was secured to the adventitia of the aorta using a 7-0 proline suture. The body of the transducer and the rest of the cannula were secured to the psoas muscle with a series of 4-0 silk sutures.

Experiment A: Effects of parity on pressor response to PE:

Protocol: After 1-day acclimatization to the telemetry cages, MAP, systolic blood pressure (SBP) and diastolic blood pressure (DBP) of conscious, unrestrained rats were continuously monitored in a stress-free environment using the PhysioTel Telemetry System (Data Sciences International) the data were later analyzed offline (Windaq, DATAQ Instruments). On the day of the experiment, baseline blood pressure was recorded for an hour. This was followed by short-term infusions (over 30 seconds) of 1, 3, 10 and 30 μg/kg of PE, a protocol which avoided acute baroreflex resetting (114). A 10-min interval was allowed between each PE dose, during which time blood pressure and HR returned to baseline values. Baroreflex sensitivity, defined as ΔHR/ΔBP, was estimated at a dose of 10 μg/kg of PE. We found that HR was most stable after BP had fallen to 80% of

its peak value. The relationship between HR and BP was thus measured at this point.

Experiment B: Effects of parity on pressor response to acute stress:

Protocol: Baseline blood pressure was recorded in conscious rats as described above. They were then exposed to acute stress by directing a jet of pressurized air towards them for 10 seconds. Changes in BP and HR were continuously recorded and changes in the MAP, SBP and DBP were compared between RB and Virgin control rats.

2.2.2 Effects of parity on the reactivity and compliance of isolated arteries:

2.2.2.1 Mesenteric arterial reactivity and compliance:

Drugs and solution: Vascular reactivity experiments were conducted in HEPES-buffered phosphate saline solution (HEPES-PSS); composition (in mmol/L): 142 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.56 $CaCl_2$, 1.18 K_2PO_4, 10 HEPES and 5.5 glucose. pH was adjusted to 7.4 with NaOH solution. Stock solutions of PE, L-NAME and meclofenamate were prepared in distilled water and stored at -40°C. Immediately before the experiments, the drugs were thawed and dissolved in HEPES-PSS to achieve the required concentration. All drugs and salts were purchased from Sigma Aldrich (Canada).

The vascular compliance experiments were conducted in calcium-Free

Dulbecco's medium; composition (in mmol/L): 15 HEPES, 15 glucose, 1 sodium pyruvate, 25 sodium bicarbonate and 1 μM EGTA with 1 g l^{-1} albumin (IgG and endotoxin free) at pH 7.4). It has previously been reported that, in the pressure myograph system, this medium helps to maintain the physiological properties of the blood vessels (127).

Vessel preparation: The rats were sacrificed by decapitation. The distal part of the small intestine and its associated vascular arcade was rapidly removed through a midline laparotomy and transferred to a silicone coated dissecting dish containing either ice-cold HEPES-PSS buffer or Dulbecco's medium. Arteries <250 μm in diameter and ~2 mm in length, were then dissected for wire myography (second order) or for pressure myography (third order) to study vascular reactivity and compliance respectively.

Experiment A: Effects of parity on mesenteric artery reactivity (Wire Myograph Study):

Stabilization of isolated arteries: Isolated mesenteric arteries were threaded on two 25 μm diameter tungsten wires (Fine wire company, California, USA) and mounted on an isometric small wire myograph station (Kent Scientific, Litchfield, California). Vessels were allowed to stabilize for 30 min in HEPES-PSS buffer under zero tension, during which time the buffer solution was changed at 10 min intervals. After stabilization, a preconditioning stretch was performed and the vessels were allowed to stabilize in HEPES-PSS buffer for another 10 min. This

was followed by generation of a baseline curve to determine resting length-tension property according to the method described Halpern W *et al.* (146). From Laplace's law, the circumference that an artery would have at a transmural pressure of 100 mmHg (L_{100}) was calculated from the exponential curve fit of tension generated vs. internal vessel circumference. Preliminary studies on second order mesenteric arteries indicated that the point on the passive-active tension characteristics curve obtained at $0.8L_{100}$ provided the maximum active tension, with the least passive tension. Real-time changes in isometric force were recorded on a computer using data-acquisition software (Windaq, DATAQ Instruments, Akron, OH).

PE sensitivity of mesenteric arteries: After stabilization the vessels were set at their optimal tensions, and concentration response curves to cumulative concentrations of PE were generated.

Contribution of endothelium on PE reactivity: To study the role of endothelium in the altered vascular reactivity, mesenteric arteries were mechanically denuded of endothelium by passing a human hair through the lumen (160), after which the constrictive response to PE was measured. Complete endothelium removal was confirmed pharmacologically by adding a single dose of methacholine (50 μmol l^{-1}) at the end of each cumulative dose regime; vessels were deemed to be endothelium-denuded if there was no relaxation after adding methacholine (160).

Effects of parity on NO and cyclooxygenase pathways: To determine which

endothelial factors were involved in the parity-induced alteration in vascular reactivity, endothelium-intact mesenteric vessels were divided into three groups: control vessels (not incubated with any drug), L-NAME-treated (incubated with L-NAME; NOS inhibitor, 100 μmol l^{-1}) and meclofenamate-treated (incubated with meclofenamate; cyclooxygenase blocker, 10 μmol l^{-1}). Vessels were incubated with L-NAME or meclofenamate for 20 min, after which (without washout) the constrictive responses to PE were measured.

Effects of parity on endothelium-dependent relaxation of mesenteric arteries: To assess endothelium-dependent vasorelaxation, isolated mesenteric vessels were first exposed to a cumulative concentration-response regime of PE to determine the concentration of PE required to produce 80% constriction (EC_{80}). The vessels were then pre-constricted with PE (EC_{80}) and methacholine concentration-response curves were completed.

Experiment B: Effects of parity on mesenteric arterial compliance (Pressure Myography Study):

The diameter-pressure relation of isolated mesenteric vessels was examined, by pressure myography (Living Systems Instrumentation, USA), using calcium-free Dulbecco's medium bubbled with 95% air–5% CO_2 (Praxair). The vessel was mounted on the inflow cannula (diameter 80-100 μm) and secured with a single fiber of a multifilament braided nylon thread. At this point the inflow stopcock was opened, and a gentle flow was allowed through the lumen of the vessel,

keeping pressure <10 mmHg for 1 minute to flush the blood and metabolites from the lumen. Flow was then stopped and the distal end of the vessel was mounted and tied onto the outflow pipette. The distal stopcock was then closed so that blind-sac (no-flow) experiments could be performed. Intraluminal pressure was briefly increased to 100 mmHg to ensure there were no leaks in the system, the system was considered to be leak-free if there was no drop in pressure during this time. The vessel chamber was slowly warmed to 37°C over 5-10 min and maintained at 37 ± 0.5°C throughout the duration of the experiment. Vessel diameter and wall/lumen ratio were displayed and measured using a CCD camera (Hitachi, Canada) and a video dimension analyzer (Living Systems Instrumentation, Burlington, VT).

Protocol: Vessels were superfused (200 ml/h) and perfused (20µl/min) with Dulbecco's medium from a reservoir, maintained at 37°C, and bubbled with 95% air and 5% CO_2. Vessels were first stabilized for 30 min at a physiological flow rate (20 µl/min) to remove the metabolites. The distal stopcock was then turned off and the vessel was stabilized for 60 min at a physiological pressure (60 mmHg). Pressure was increased and monitored in a stepwise manner from 20 to 140 mmHg (20 mmHg every 5 min) using the Pressure Servo Unit (Living Systems Instrumentation, Burlington, VT) attached to a calibrated pressure transducer at the inflow stopcock.

Arterial compliance was calculated as the ratio of *Δ internal diameter: Δ pressure*, in the physiological range of 80-120 mmHg intraluminal pressure.

Circumferential strain was calculated as $\varepsilon = (D_1 - D_o)/D_o$, where D_1 is the observed lumen diameter for a given intraluminal pressure and D_o is the original diameter measured at 20mmHg intraluminal pressure. Circumferential stress was calculated as $\sigma = (PD)/(2WT)$, where P is the intraluminal pressure and D and WT are the lumen diameter and wall thickness, respectively. Pressure was converted from mmHg to dyn/cm^2 (1mmHg=1.334×10^{3}dyn/cm^2).

2.2.2.2 Effects of parity on femoral artery reactivity:

Another set of rats were decapitated, and segments of femoral artery (~2 cm axial length) were dissected out (0–4 °C) in HEPES-PSS buffer and mounted on two wires (25 μm) of a wire myograph system (Kent Scientific, Litchfield, CA, USA). The vessels were then stabilized and the constrictive responses to a cumulative concentration regime of PE were measured as described above.

2.2.3 Effects of parity on histology of aorta and mesenteric and renal arteries:

Segments of mesenteric and renal arteries and aorta were dissected out from RB (n=8) and Virgin (n=5) rats, and fixed with 10% formalin. The tissues were then processed for histological evaluation using hematoxylin and eosin staining (Health Sciences Laboratory Animal Services, University of Alberta). Hematoxylin stained the nucleus as blue and eosin stained the cytoplasm and connective tissue as red.

2.3 Effects Of Parity On Venous System:

2.3.1 Effect of parity on whole body venous tone response to NA (MCFP Study):

General surgical procedures: The rats were anesthetized using halothane. During surgery, the rats were placed on Deltaphase isothermic heating pad (Braintree Scientific, Inc., Mass., USA) to maintain the body temperature at ~37°C. At least 5hr, but less than 8hr, were allowed to elapse before MCFP was measured. This allowed for recovery from surgery and anesthesia, but minimized the risk of thrombogenesis from the intracardiac balloon.

Implantation of femoral cannulae: An approximately 1.5 cm long medial incision was made along the thigh of both legs, and the femoral arteries and veins were exposed. Polyethylene cannulae (PE 50, ID: 0.58 mm, OD: 0.97 mm; VWR International, Mississauga, Ontario, Canada) were implanted in the vessels and secured with 4-0 silk. Three polyethylene cannulae were implanted during the surgery: 1) right femoral vein to infuse drugs, 2) inferior vena cava via left femoral vein to measure CVP and 3) left iliac artery to measure systemic blood pressure and HR. All cannulae were filled with heparinized normal saline (25 IU/ml) and tunneled subcutaneously to the midscapular region of the back.

Implantation of balloon in the right atrium: An approximately ~ 1.5 cm long incision was made above the right jugular vein. The jugular vein was bluntly freed

from the surrounding tissue. Using retraction sutures, blood flow was stopped and a tiny hole was punctured into the vessel using fine forceps. A saline-filled balloon-tipped catheter was pushed through the hole into the right jugular vein towards the right atrium (Figure 6). The tip of the balloon was then pushed ~2cm, a maneuver which caused an instantaneous fluctuation in HR and MAP. The balloon was then pushed ~0.5cm further in. The cannula of the balloon was fixed to the neck muscles using 4-0 silk sutures. Inflation of the balloon, when correctly positioned, caused an instantaneous reduction in blood pressure and HR accompanied by an increase in CVP (34). The cannula was tunneled subcutaneously to the midscapular region of the back and exteriorized and sealed.

Protocol (MCFP response to NA): Unrestrained animals were placed in a small cage and allowed to eat and drink freely. Central venous and arterial cannulae were connected to Gould Statham Pressure Transducers (Model: 13-4615-50, Gould Inc., Cleveland, OH) and Gould Statham Pressure Processors (Model: 13-4615-52, Gould Inc., Cleveland, OH) respectively to amplify pressure signals, and to determine MAP and HR. These signals were then digitized (DI-205, Dataq Instruments, Akron, OH) and systemic arterial pressure, MAP, HR and CVP were recorded online using WINDAQ data acquisition software (Dataq Instruments, Akron, OH). Baseline MAP, CVP and HR were recorded for 20 minutes. MCFP was calculated using the formula MCFP= VPP + K (FAP-VPP) (165), where VPP and FAP represents the final arterial pressure and the venous plateau pressure respectively; K is a constant representing the ratio of venous and arterial

compliance (equals 1/60) (165). Venous and arterial pressures were measured during circulatory arrest induced by inflating the intracardiac balloon for ~ 5-7 sec, until CVP had plateaued (Figure 7). This technique to measure MCFP was first developed by Yomomoto *et al* in 1980. They also showed that it is critical to measure MCFP response within 10 sec of balloon inflation as stopping the circulation for longer causes a centrally mediated increase in CVP. We used the non-specific adrenergic agonist NA, because it has been reported that the rat venous side of splanchnic vascular bed constricts predominately to α-2 adrenergic agonists (167). In order to minimize the effects of NA on the heart, β-adrenergic receptors were blocked with propranolol 1mg/kg i.v. Following a 10 min stabilization period, baseline MAP, HR, CVP and MCFP measurements were made. After a further 10 min recovery period, dose-response curves to NA (1, 3, 10, 30 and 100×10^{-9} mol/kg/min) were constructed. At each dose, it took ~3min for the MAP to plateau, at which point MAP, HR, CVP and MCFP measurements were made. A 10 min recovery period was allowed between each dose of NA. This protocol has previously been used to make repeated measurements of venous tone (165).

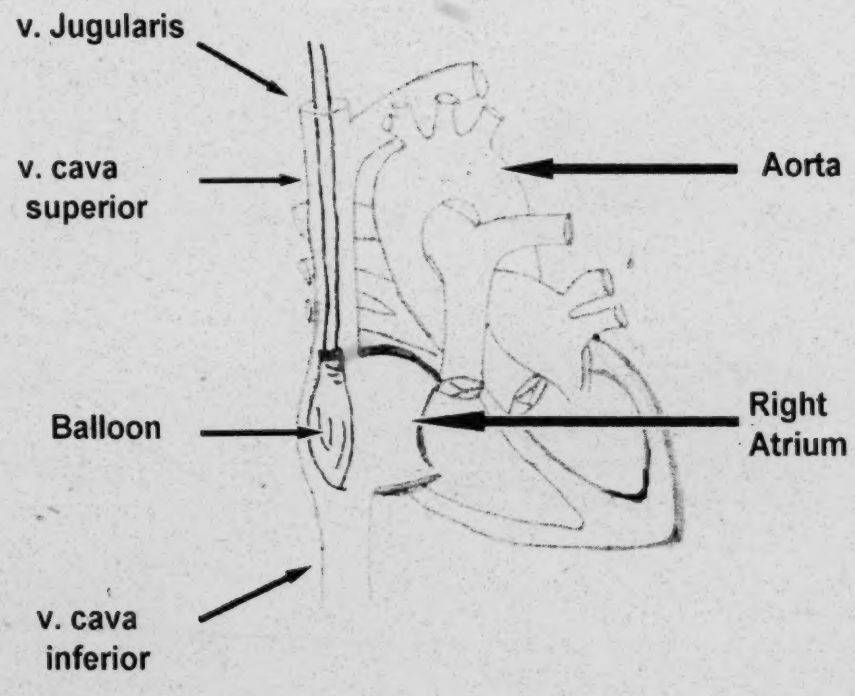

Figure 6: Position of saline-filled balloon in right atrium for mean circulatory filling pressure measurements.

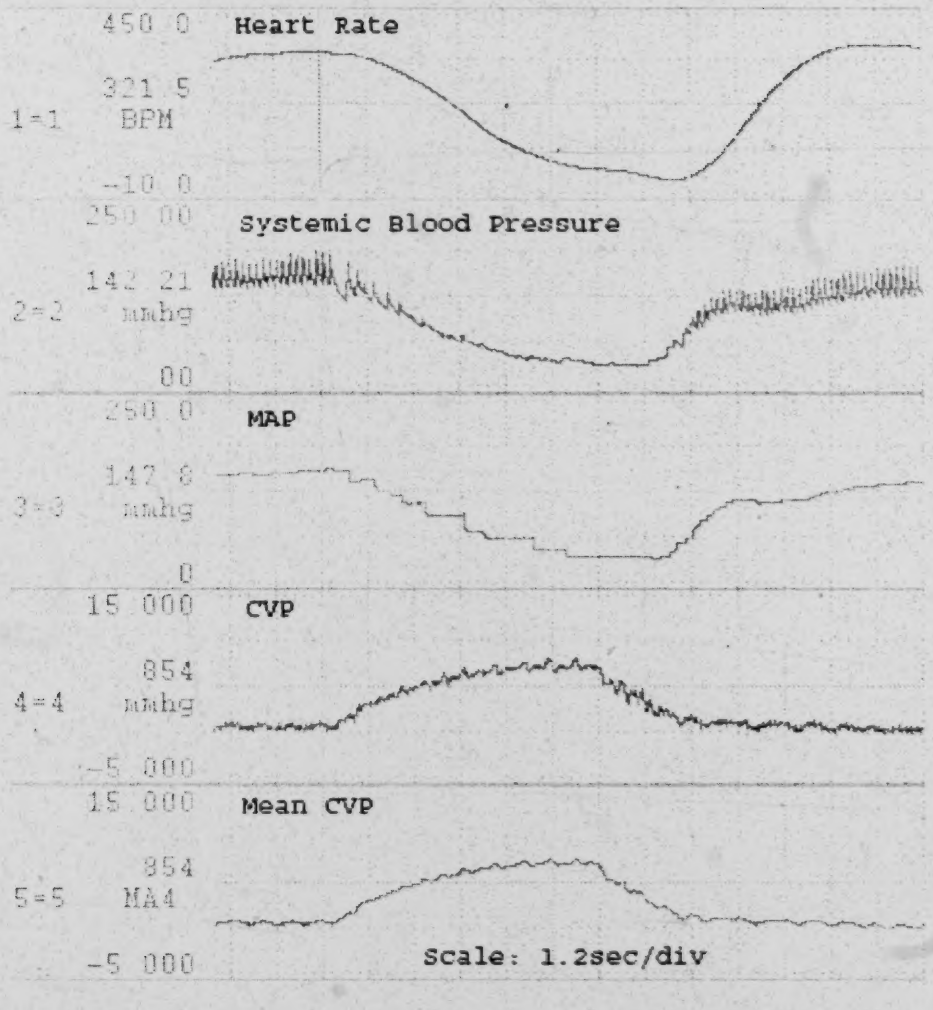

Figure 7: Trace of heart rate, systemic blood pressure and central venous pressure (CVP) while performing circulatory arrest to measure mean circulatory filling pressure. MAP and mean CVP were calculated in real-time using Gould ™ Pressure Processor.

2.3.2 Effects of parity on blood volume and blood pressure response to volume loading:

General surgical considerations: Rats were anesthetized with sodium pentobarbital (62 mg/kg body wt. i.p.), followed by atropine (0.1 ml, 0.4 mg/ml s.c.). Buprenorphine (0.01 mg/kg s.c.) was given after the completion of surgery. A non-occlusive cannula (ID: 0.51-mm, OD: 0.94-mm; Silastic, Dow Corning, Midland, USA) was implanted into the inferior vena cava (i.v.c.; as described in 2.2.1) for infusing the volume load and for taking blood samples. Another Silastic™ cannula of the same diameter was implanted in the jugular vein to infuse dye for blood volume measurement. Briefly, an approximately ~ 1.5 cm long incision was made above the right jugular vein. The jugular vein was bluntly freed from the surrounding tissue. Using retraction sutures, blood flow was stopped and a tiny hole was punctured into the vessel using fine forceps. A cannula (Silastic, ID: 0.51-mm, OD: 0.94-mm) was pushed through the hole into the vein, and advanced towards the heart (~ 1 cm). The cannula was fixed to the neck muscles and clavicle bone using 4-0 silk sutures. A telemetric pressure transmitter (PAC-40, Data Sciences International, Minnesota, USA) was implanted in the abdominal aorta (as described in 2.2.1) to measure systemic blood pressure. Animals were allowed to recover for one week from surgery and to regain their preoperative body weight.

Experiment A: Plasma volume measurements:

Protocol: Plasma volume was determined by the Evans blue dye dilution method (106). Briefly, initial blood samples (0.25 ml) were taken. A solution (0.3 ml, 0.5% W/V) of Evans blue (Baker Chemical, Phillipsburg, NJ) was injected in the cannula implanted in the jugular vein, which was then flushed with 0.2 ml saline. Blood samples (0.15 ml) were taken from the i.v.c. cannula at 10, 20, 30, 40, and 60 min, and the volumes replaced with isotonic saline infused through the same cannula. The blood samples were transferred to heparinized Microvette (Sarstedt, Aktiengesellschaft & Co, Numbrecht Germany), and centrifuged to separate the plasma and red blood cells. Hematocrit was measured and the plasma samples (50 µl) were then diluted in 950 µl saline to measure the absorbance at 605 µm on a spectrophotometer (LKB Biochrom, model 4049, Cambridge, UK). The readings were compared with standards obtained by adding 0, 1, and 2 µl of the 0.5% Evans blue solution to 50 µl initial plasma sample plus 950 µl saline. The plasma volume and blood volume were determined by extrapolation back to time zero.

Experiment B: Blood pressure response to volume loading:

Protocol: After one day acclimatization to the metabolism cages, baseline MAP was recorded for 1hr in conscious, unrestrained animals (PhysioTel Telemetric System, Data Sciences International, Minnesota, USA). The rats were then challenged with a volume overload by infusing Pentaspan (2ml/100g/min, 10% Pentaspan in 0.9% NaCl, DuPont Pharma Inc, Canadian Blood Bank services). These experiments were always conducted at the same time of day (10.00-

12.00am). Data were later analyzed offline (Windaq, DATAQ Instruments, Akron, USA).

2.3.3 Effects of parity on reactivity and compliance of isolated veins:

Drugs and solution: Venous reactivity experiments were conducted in HEPES-PSS; composition (in mmol/L): 142 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.56 $CaCl_2$, 1.18 K_2PO_4, 10 HEPES and 5.5 glucose. pH was adjusted to 7.4 with NaOH. Stock solution of PE and NA were prepared in distilled water and stored at -40°C. Immediately before the experiments, the drugs were thawed and dissolved in HEPES-PSS to achieve the required concentration. All drugs and salts were purchased from Sigma Aldrich (Canada).

2.3.3.1 Mesenteric venous reactivity and compliance:

Mesenteric venous reactivity was first studied using wire myography (similar protocol followed as in 2.3.3.2). However, due to low maximum tension produced using wire myography and low consistency in the vascular responses (Appendix B; Figure 30), mesenteric venous reactivity experiment were repeated using pressure myography, which has been described as a more "physiological" technique to measure vascular reactivity (61).

Experiment A: Effects of parity on mesenteric venous reactivity: Preparation of

isolated veins:

The rats were sacrificed by decapitation. The distal part of the small intestine and its associated vascular arcade were rapidly removed through a midline laparotomy and transferred to a silicone coated dissecting dish containing either ice-cold (0–4 °C) HEPES-PSS buffer or calcium-free Dulbecco's medium. Second order mesenteric veins ~2 mm in length, were dissected in cold HEPES and used to study venous reactivity. Third order veins were dissected in Dulbecco's medium and used to study venous compliance.

Pressure myography of isolated veins: Isolated veins were mounted on the cannulae (180-200 μm) of a small vessel myograph chamber (CH/2/SH, Living Systems Instrumentation, Burlington, USA) and the experiments were performed using blind-sac (no-flow) technique. Leaks were tested by increasing the intraluminal pressure to 5 mmHg using a pressure servo system (PS200/Q, Living Systems Instrumentation, Burlington, USA), and the system was considered to be leak-free if there was no drop in pressure. During the equilibration period, the vessels were first exposed to an intraluminal physiological flow rate (2 μl/min) for 10 min to flush out metabolites, and then stabilized at no flow condition at 5mmHg for 30 minute. Temperature of the vessel chamber was maintained at 37 ± 0.5°C throughout the duration of the experiment using a temperature servo controller (Living Systems Instrumentation, Burlington, USA).

Protocol: After stabilization, the mesenteric veins were incubated with

propranolol (10^{-6} M) for 15 min, in order to block the effect of NA on venous β-adrenergic receptors. The vessels were then exposed to a cumulative concentration-response regime of NA (1×10^{-9} to 1×10^{-6} M) and the changes in diameter were measured using a CCD camera (Sony, Japan) and a video dimension analyzer (Living Systems Instrumentation, Burlington, USA). EC_{50}, the concentration required to produce 50% of the maximum response, was calculated from sigmoidal plots (SigmaPlot, Systat Software Inc, CA) of concentration-response curves (% change of initial diameter) constructed for each individual vessel.

Experiment B: Effects of parity on mesenteric venous compliance:

The vascular compliance experiments were conducted in calcium-free Dulbecco's medium; composition (in mmol/L): 15 HEPES, 15 glucose, 1 sodium pyruvate, 25 sodium bicarbonate and 1 μM EGTA with 1 g l^{-1} albumin (IgG and endotoxin free) at pH 7.4).

The diameter-pressure relation of small mesenteric vessels was examined, by pressure myography (Living Systems Instrumentation, Burlington USA), using calcium-free medium as previously described (21). Venous compliance was calculated as the ratio of *Δ internal diameter: Δ pressure*, in the physiological range of 4-8mm Hg intraluminal pressure. Circumferential strain was calculated as $\varepsilon = (D_I - D_o)/D_o$, where D_I is the observed lumen diameter for a given intraluminal pressure and D_o is the original diameter measured at 2mmHg

intraluminal pressure. Circumferential stress was calculated as $\sigma = (PD)/(2WT)$, where P is the intraluminal pressure and D and WT are the lumen diameter and wall thickness, respectively. Pressure was converted from mmHg to dyn/cm^2 ($1mmHg=1.334\times10^3 dyn/cm^2$).

2.3.3.2 Saphenous venous reactivity:

Preparation of isolated veins: The rats were sacrificed by decapitation and a segment of saphenous vein, ~2 mm in length, was then isolated and transferred to a wire myograph system (Kent Scientific, Litchfield, CA, USA) for measurement of the constrictive responses to PE .

Wire myography of isolated veins: Isolated saphenous veins were threaded on two 25 μm diameter tungsten wires (Fine wire company, California, USA) and mounted on an isometric small wire myograph station (Kent Scientific, Litchfield, California). Vessels were allowed to stabilize for 30 min in HEPES-PSS buffer under zero tension, during which time the buffer solution was changed at 10-min intervals. After stabilization, a preconditioning stretch was performed and the vessels were allowed to stabilize in HEPES-PSS buffer for another 10 min. This was followed by generation of a baseline curve to determine resting length-tension property according to the method described Halpern W *et al.* (146). From Laplace's law, the circumference that an artery would have at a transmural pressure of 10 mmHg (L_{10}) was calculated from the exponential curve fit of

tension generated vs. internal vessel circumference. Preliminary studies on saphenous veins indicated that the point on the passive-active tension characteristics curve obtained at $0.8L_{10}$ provided the maximum active tension, with the least passive tension. Real-time changes in isometric force were recorded on a computer using data-acquisition software (Windaq, DATAQ Instruments, Akron, OH).

Protocol: After stabilization the vessels were then set at their optimal tensions and concentration response curves to cumulative concentrations of PE were generated.

2.4 Effects Of Parity On Serum Estrogen Levels:

Serum estradiol was measured using an Ultra-Sensitive Estradiol radioimmunoassay (RIA) kit with detection limit of 2.2 pg ml^{-1} (Diagnostics Systems laboratories). All samples were run in duplicate and the standard protocol supplied with the RIA kit was followed.

2.5 Statistical Analysis:

Between groups variation (RB vs. Virgins) was assessed using repeated measures two-way ANOVA, followed by post hoc analysis with the Student-Newmans-Keuls test (Figure 8, 9, 16 (A), 20 and 22 (A)). In the stress response study (Figure 10 and 11), each data point represents the mean changes in the blood pressure over a period of 5 seconds and the difference of each point between the groups was considered statistically significant at $P<0.05$ using one-tailed t-test (one-tailed t-test was used because based on the results of the PE pressor response study we hypothesized that RB will have a greater pressor response to stress than virgin rats, i.e. we expected that the changes in blood pressure response to acute stress will not be similar).

EC_{50}, the concentration required to produce 50% of the maximum response, was calculated from sigmoidal plots (SigmaPlot, Systat Software Inc, CA) of concentration-response curves constructed for each individual vessel. For simple comparisons between two sets of data such as animal weight, EC_{50}, MAP or compliance we used two tailed Students t-test for unpaired data. For comparison between control and drug treatment within groups (RB and Virgin), two-way ANOVA was used (Table 3). In the mesenteric venous reactivity study (2.3.3.1; Experiment A), where we predicted that EC_{50} of veins from RB would be less than that from virgin rats, we used a one-tailed t-test for unpaired data. All

data are presented as mean ± SE of mean. All results were considered statistically significant at $P<0.05$.

3 RESULTS

The RB rats (390.6± 4.47g, n=69) were slightly heavier than the age-matched Virgin rats (346.6± 4.45g, n=78); $P<0.05$.

3.1 Arterial System:

3.1.1 Effects of parity on pressor response to PE and acute stress:

Effects of parity on baseline cardiovascular parameters: There was no significant difference between the resting MAP of the RB and Virgin rats, although it tended to be lower in the parous rats (RB: 98.7±2.4 mmHg, n=12 vs. Virgin: 104.3±2.1 mmHg, n=12; P=0.09). Nor were there any significant differences between the groups with regard to systolic (SBP; RB: 117.0±2.9 mmHg, n=12 vs. Virgin: 119.5±2.7 mmHg, n=12) or diastolic (DBP; RB: 86.8±2.3 mmHg, n=12 vs. Virgin: 91.7±2.0 mmHg, n=12) blood pressures.

Experiment A: Effects of parity on pressor response to PE:

The pressor response to PE was significantly greater in RB animals than in the age-matched Virgin control rats (Figure 8). This was also associated with a greater increase in SBP and DBP in RB rats as compared to the Virgins (Figure 9). There was no significant difference in the baroreflex sensitivity between the two groups ($\Delta HR/\Delta BP$; RB: -2.76±0.86 bpm/mmHg, n=6 vs. Virgin: -2.80±0.48 bpm/mmHg, n=7; $P \geq 0.05$).

Experiment B: Effects of parity on pressor response to acute stress:

Acute stress, induced by a jet of pressurized air, elicited an immediate increase in MAP, which was significantly greater in the RB animals compared to Virgins (Figure 10A). There was also a significant greater increase in SBP and DBP in RB animals compared to Virgin animals (Figure 11). However, there was no significant difference in the stress induced changes in HR of the RB and Virgin rats (Figure 10B).

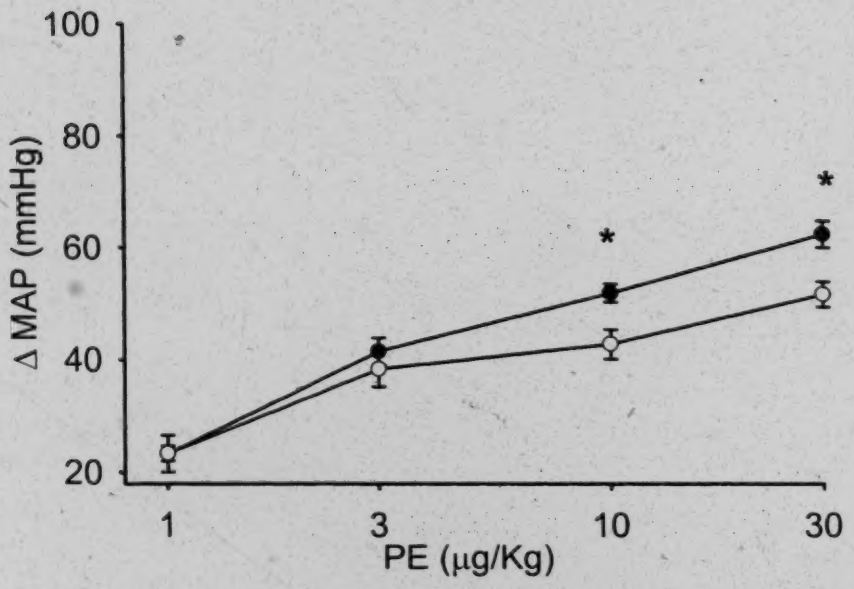

Figure 8: Effect of parity on changes in mean arterial pressure (MAP) to intravenous phenylephrine (PE) administration in conscious rats. Repeatedly Bred rats: closed circles (n= 6); Virgins: open circles (n=7). Vertical lines delineate standard error of mean. *, $P<0.05$, significant difference between Repeatedly Bred and Virgin rats.

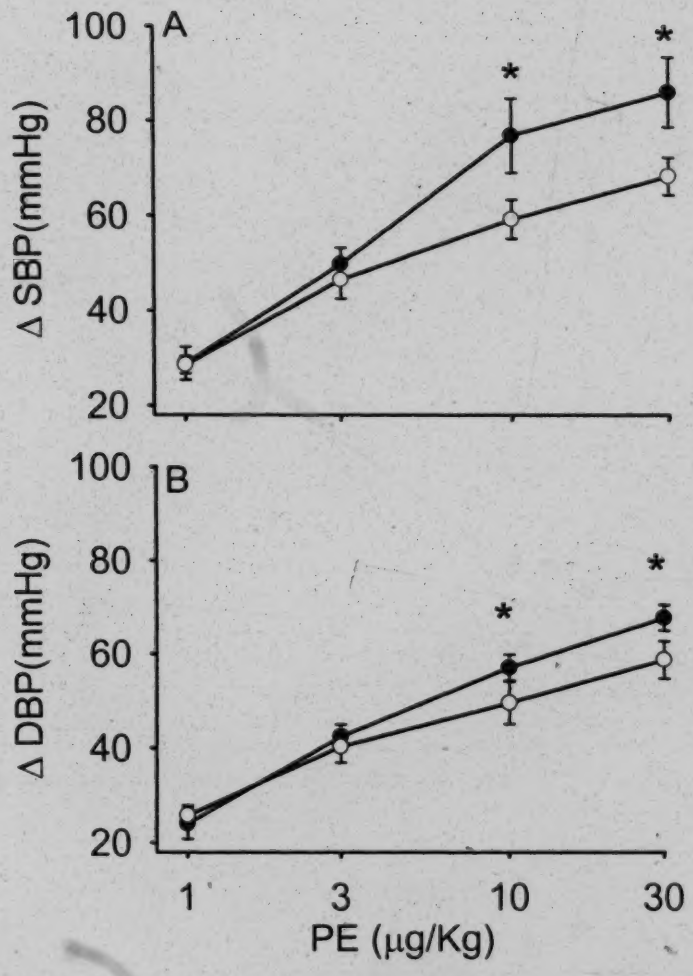

Figure 9: Effect of parity on changes in A) systolic blood pressure (SBP) and B) diastolic blood pressure (DBP) to intravenous phenylephrine (PE) administration in conscious rats. Repeatedly Bred rats: closed circles (n= 6); Virgins: open circles (n=7). Vertical lines delineate standard error of mean. *, $P<0.05$, significant difference between Repeatedly Bred and Virgin rats.

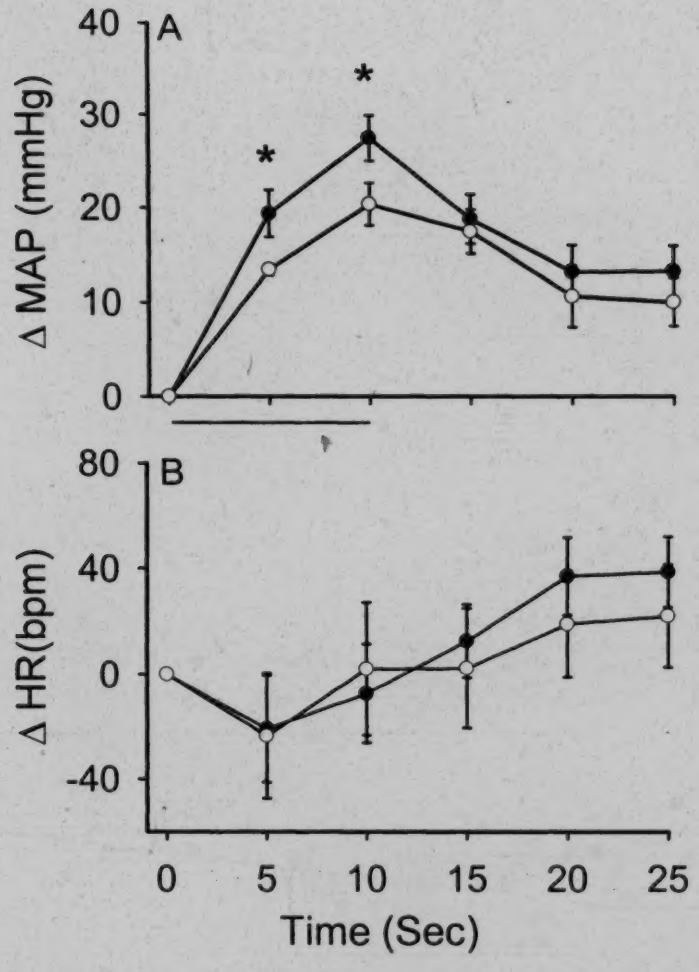

Figure 10: Effect of parity on changes in A) mean arterial pressure (MAP) and B) heart rate (HR) responses to stress (10 sec air jet) in conscious rats. Repeatedly Bred rats: closed circles (n=6); Virgins: open circles (n=5). The horizontal bar shows the period during which stress was administered (10 sec). Vertical lines delineate standard error of mean. *, $P<0.05$, significant difference between Repeatedly Bred and Virgin rats.

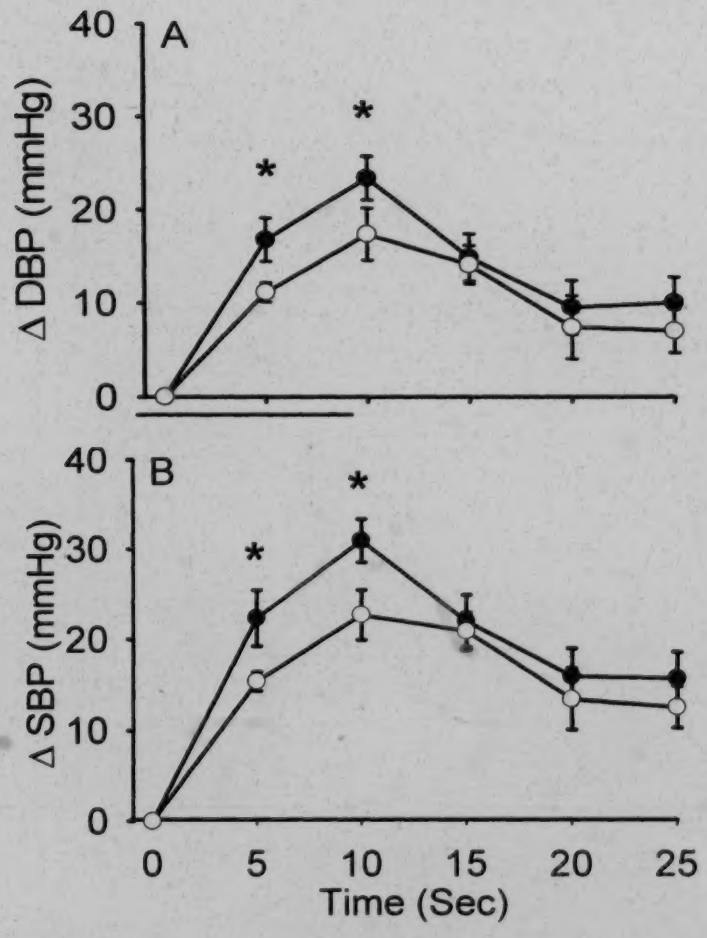

Figure 11: Effect of parity on changes in A) diastolic blood pressure (DBP) and B) systolic blood pressure (SBP) responses to stress (10 sec air jet) in conscious rats. Repeatedly Bred rats: closed circles (n=6); Virgins: open circles (n=5). The horizontal bar shows the period during which stress was administered (10 sec). Vertical lines delineate standard error of mean. *, $P<0.05$, significant difference between Repeatedly Bred and Virgin rats.

3.1.2 Effects of parity on reactivity and compliance of isolated arteries:

3.1.2.1 Mesenteric artery reactivity and compliance:

Experiment A: Effects of parity on mesenteric arterial reactivity (Wire Myograph Study):

Mesenteric arteries from RB were more sensitive to PE than those from Virgin animals (Figure 12), although the maximum response did not differ (Table 1). Removal of endothelium shifted the concentration-response curves of both the RB and Virgin rats leftwards, such that the difference between the two groups was abolished (Figure 12). There was no difference between their maximum responses (Table 1).

Incubation of mesenteric arteries with L-NAME or meclofenamate has no effect on baseline tension of either RB or Virgin rats (Table 2). Pretreatment with L-NAME did not alter the maximum PE-induced vasoconstriction of the mesenteric arteries from either RB or Virgin rats. However, the concentration response curves from both groups were shifted leftwards, such that the difference in PE sensitivity between them was abolished (Figure 13 and Table 3). Incubation of mesenteric arteries with meclofenamate significantly shifted the PE concentration response curve to the right in RB but not in Virgin rats (Figure 14 and Table 3). Like L-NAME, meclofenamate did not alter the maximum response to PE (Table 3).

Although endothelium-dependent relaxation to methacholine tended to be attenuated in RB compared to Virgins, it failed to reach significance (Figure 15; RB EC_{50}: $5.37\pm1.29\times10^{-7}$M, n=8 vs. Virgins EC_{50}: $3.64\pm1.29\times10^{-7}$M, n=9; P=0.360).

Experiment B: Effects of parity on mesenteric arterial compliance (Pressure Myography Study):

In both RB and Virgin rats, the diameter of small mesenteric arteries increased with increasing transluminal pressure. However, within the physiological pressure range (80-120 mmHg), the ratio of Δ diameter: Δ pressure was significantly lower in arteries from RB than from age-matched Virgin control rats (Figure 16A; RB: 0.24±0.04mm/mmHg, n=6 vs. Virgins: 0.63±0.06 mm/mmHg, n=6; P=0.05). There was no significant difference between the wall: lumen ratio, measured at 100 mmHg of transmural pressure, of the two groups (RB: 0.27±0.01, n=6 vs. Virgins: 0.28±0.08, n=6). The stress-strain relationship of Mesenteric arteries from RB was positioned leftwards than that of arteries from the Virgin rats (Figure 16B), suggesting that arteries from RB are stiffer than that from Virgin rats.

3.1.2.2 ***Effects of parity on femoral artery reactivity (Wire Myograph Study):***

There was no significant difference between the sensitivity of femoral arteries from RB compared with Virgin rats (Figure 17; RB EC_{50}: $2.59\pm0.20\times10^{-6}$M, n=8 vs. Virgins EC_{50}: $2.80\pm1.78\times10^{-6}$M, n=10; P=0.459).

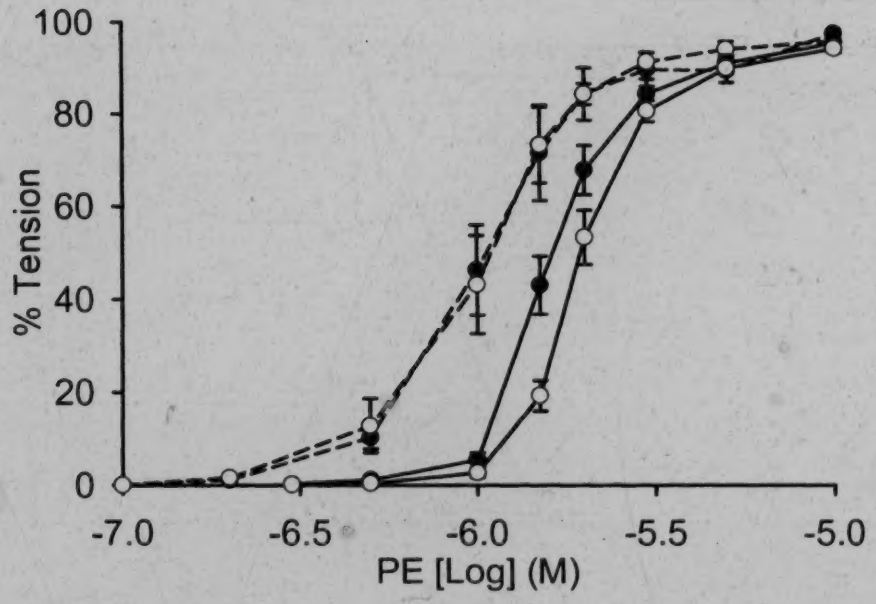

Figure 12: Concentration-response curves of phenylephrine (PE) on small mesenteric arteries. Effect of removing endothelium: intact (solid line) and endothelium-denuded (dashed line) small mesenteric arteries from Repeatedly Bred (closed circles, n=10) and Virgin (open circles, n=14) rats. Change in the tension is expressed as percentage of maximum response to PE. Vertical lines delineate standard error of mean.

Table 1: EC_{50} and maximum responses of intact (E.I.) and endothelium-denuded (E.D.) small mesenteric arteries from Repeatedly Bred (RB) and Virgin rats.

	RB		Virgin	
	E.I. (n=10)	E.D. (n=5)	E.I. (n=14)	E.D(n=6)
EC_{50} (10^{-6}M)	1.58±0.08*	1.05±0.23	2.05±0.09	1.08±0.14
Max Tension ($mN \cdot mm^{-1}$)	0.38±0.01	0.31±0.03	0.38±0.01	0.32±0.03

Date presented as mean ± standard error of mean. *, $P<0.05$, significant difference between RB and Virgin rats.

Table 2: Baseline tension of small mesenteric arteries from Repeatedly Bred (RB) and Virgin rats before and after pre-treatment with L-NAME and meclofenamate (Meclo).

	RB		Virgin	
Tension ($mN \cdot mm^{-1}$)	L-NAME (n=6)	Meclo (n=7)	L-NAME (n=5)	Meclo (n=6)
Before	0.075±0.013	0.083±0.008	0.084±0.013	0.062±0.065
After	0.073±0.012	0.081±0.009	0.083±0.013	0.060±0.063

Date presented as mean ± standard error of mean.

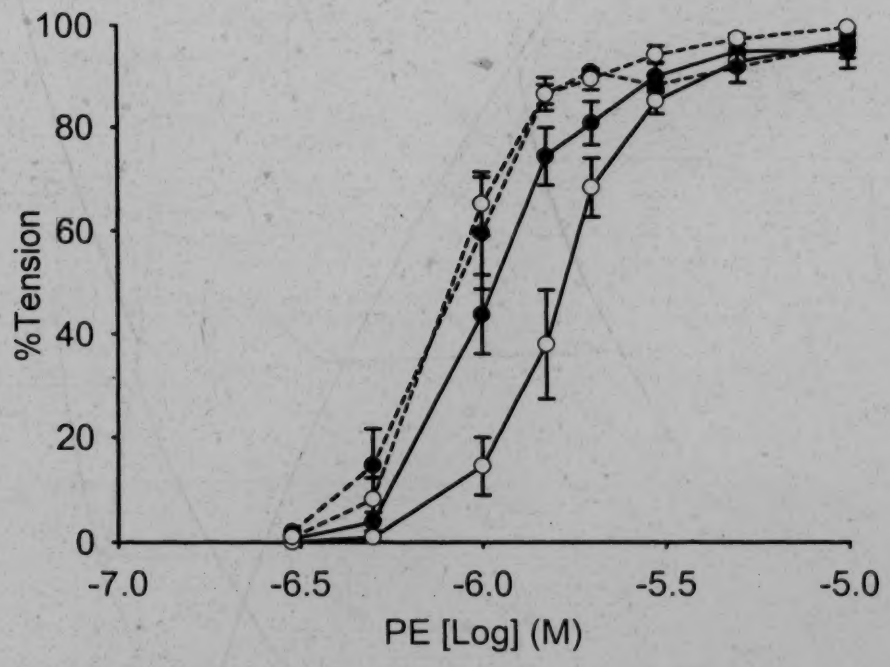

Figure 13: Concentration-response curves of phenylephrine (PE) on small mesenteric arteries. Effect of NOS inhibition: control (solid line) and L-NAME (100μM) treated (dashed line) small mesenteric arteries from Repeatedly Bred (closed circles, n=7) and Virgin (open circles, n=7) rats. Change in the tension is expressed as percentage of maximum response to PE. Vertical lines delineate standard error of mean.

Table 3: EC_{50} and maximum responses of small mesenteric arteries from Repeatedly Bred (RB) and Virgin rats with and without pre-treated with L-NAME and meclofenamate (Meclo).

	RB			Virgin		
	Control (n=7)	L-NAME (n=6)	Meclo (n=7)	Control (n=7)	L-NAME (n=5)	Meclo (n=6)
EC_{50} (10^{-6}M)	1.06 ±0.09*	0.88 ±0.1	2.33 ±0.43†	1.63 ±0.13	0.89 ±0.04†	2.31 ±0.22
Max Tension ($mN \cdot mm^{-1}$)	0.36 ±0.02	0.35 ±0.02	0.34 ±0.01	0.32 ±0.01	0.33 ±0.03	0.32 ±0.02

Data presented as mean ± standard error of mean.*, significant difference between RB and Virgin rats. †, significant difference between treatment and control within groups (RB or Virgin) $P<0.05$

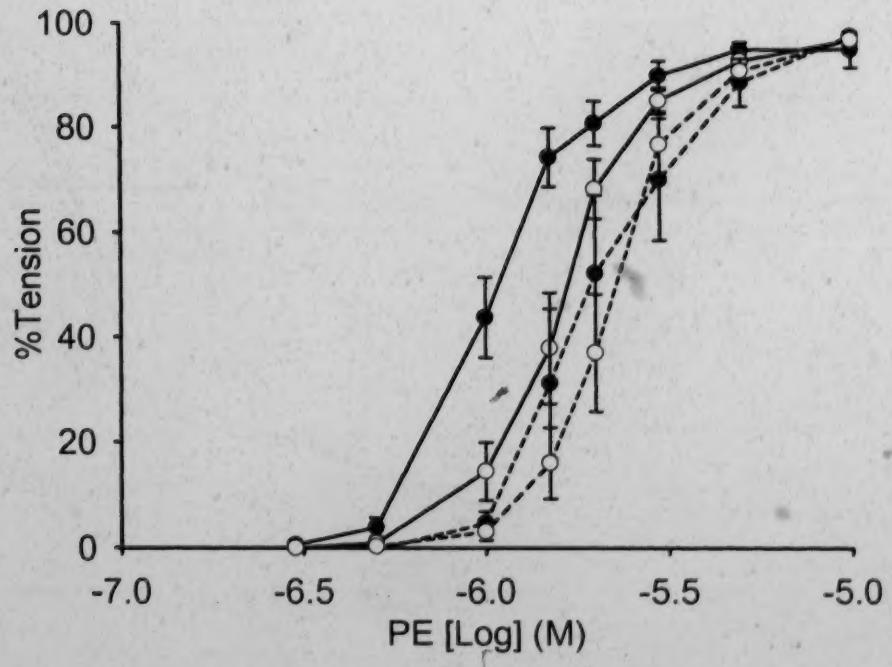

Figure 14: Concentration-response curves of phenylephrine (PE) on small mesenteric arteries. Effect of blocking cyclooxygenase: Control (solid line) and meclofenamate (10μM)) pre-treated (dashed line) small mesenteric arteries from Repeatedly Bred (closed circles, n=7) and Virgin (open circles, n=7) rats. Change in the tension is expressed as percentage of maximum response to PE. Vertical lines delineate standard error of mean.

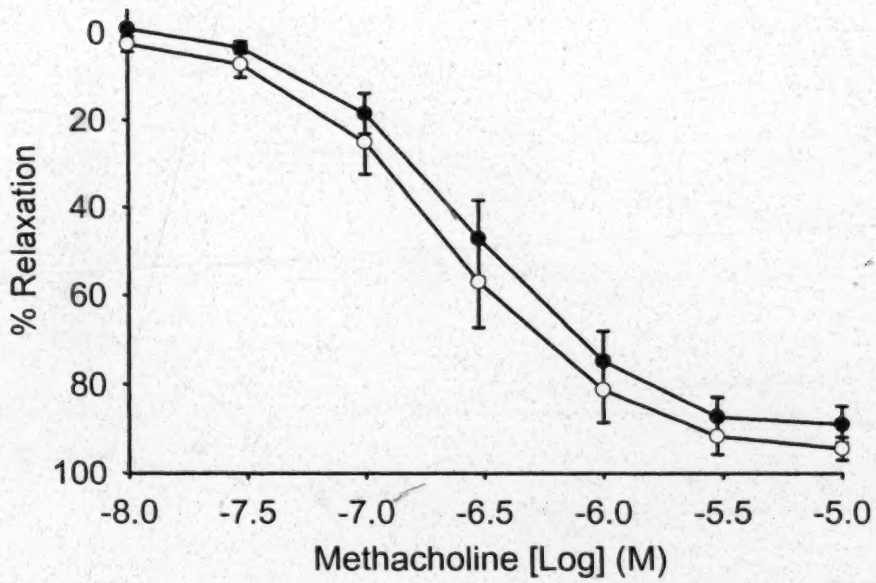

Figure 15: Concentration-response curves of methacholine on small mesenteric arteries from Repeatedly Bred (closed circles, n=8) and Virgin rats (open circles, n=9). Change in the tension is expressed as percentage relaxation to methacholine. Vertical lines delineate standard error of mean.

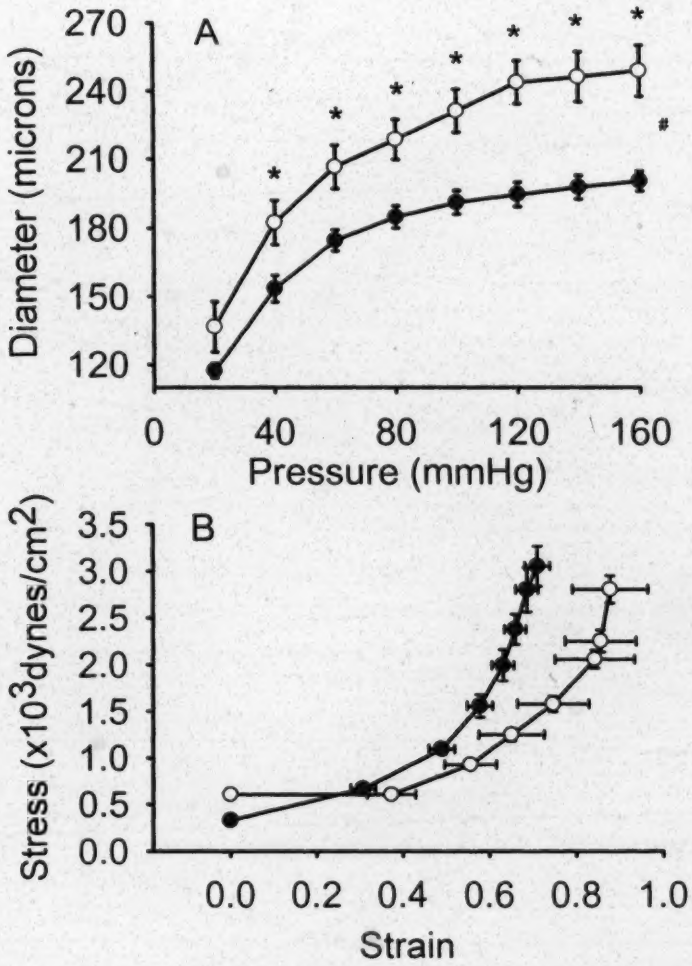

Figure 16: Effect of parity on change in A) diameter of small mesenteric arteries as a function of pressure and B) stress-strain relationship in calcium free medium. Repeatedly Bred (closed circles, n=6) and Virgin rats (open circles, n=6). Vertical lines delineate standard error of mean. *, $P<0.05$, significant difference between Repeatedly Bred and Virgin rats. (Experiments conducted with the help of Dr. Zoë Brookes).

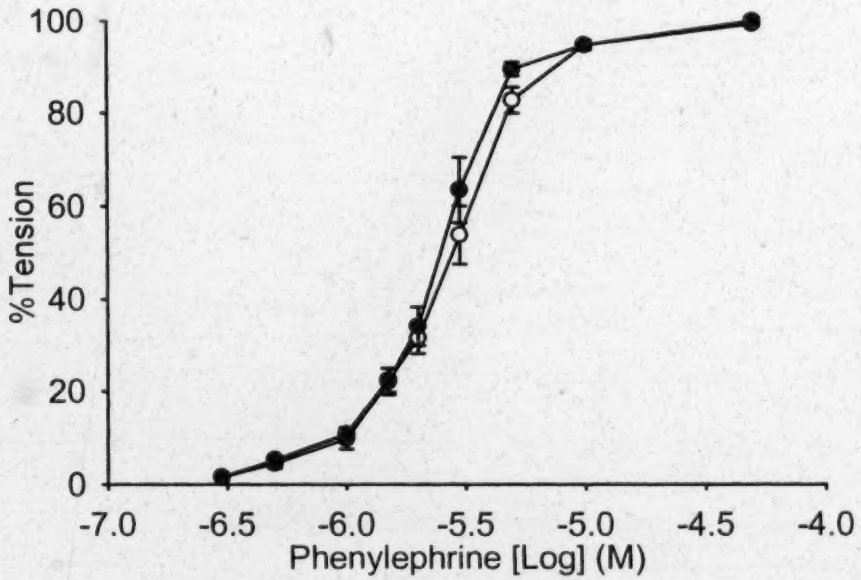

Figure 17: Concentration-response curves of phenylephrine (PE) on femoral arteries from Repeatedly Bred (closed circles, n=8) and Virgin (open circles, n=10) rats. Change in the tension is expressed as percentage of maximum response to PE. Vertical lines delineate standard error of mean.

3.1.3 Effects of parity on the histology of aorta and mesenteric and renal arteries:

There were no atherosclerotic lesions identified in any of the vessel from either the RB or Virgin rats.

3.2 Venous System:

3.2.1 Effect of parity on venous tone response to NA (MCFP Study):

There was no difference in baseline MCFP between the two groups although it tended to be lower in RB (5.7±0.8mmHg, n=6) than Virgins (7.5±0.7mmHg, n=6) rats (P=0.12). Baseline MAP and HR were also not different between the two groups. Two different protocols were used in the MCFP and volume loading studies; in the former (MCFP), MAP and HR were measured only 5hr after anesthesia and surgery animals, whereas animal in the latter study (volume loading) were allowed to recover for at least one week after surgery. We have therefore reported the baseline parameters separately in Table 4. There were no significant differences between resting MAP or HR of the RB and Virgin rats. NA caused a dose-dependent increase in both MCFP and MAP in RB and aged-matched Virgins control rats (Figure 18 and 19). However, there was a greater dose-related increase in MCFP in the RB rats compared with the Virgin rats as reflected by the greater sensitivity of MCFP response to NA in RB rats (Figure 18; RB: ED_{50} = 3.1±0.5 x 10^{-9}mol.kg^{-1}.min^{-1}, n=6 vs. Virgin: ED_{50} = 12.1±2.7 x 10^{-9}mol.kg^{-1}.min^{-1}, n=6; P<0.05). There was also a higher sensitivity of blood pressure response to NA in the RB rats than in the Virgins (Figure 19; RB: ED_{50} = 3.6±0.7 x 10^{-9} mol.kg^{-1}.min^{-1}, n=6 vs. Virgin: ED_{50} = 6.2±0.4 x 10^{-9} mol.kg^{-1}.min^{-1}, n=6; P<0.05). There was no significant difference in the baroreflex

sensitivity between RB (-1.23±0.20 bpm/mmHg, n=6) and Virgins (-1.59±0.30 bpm/mmHg, n=6) (P=0.35).

3.2.2 Effects of parity on blood volume and blood pressure response to volume loading:

Experiment A: Plasma volume measurements:

Blood volume was lower in the RB compared to Virgins rats (RB: 5.9±0.1 ml/100g, n=4 vs. Virgins: 6.9±0.3 ml/100g, n=5; P=0.025).

Experiment B: Blood pressure response to volume loading:

There were no significant differences between resting MAP or HR of the RB and Virgin rats (Table 4). There was a greater increase in MAP to volume loading in RB animals than aged-matched Virgins control rats (Figure 20A). Although the changes in HR in the animals from both the groups were similar, there was a significant transient increase from baseline in the RB rats but not in the Virgins (Figure 20B).

Table 4: Baseline mean arterial pressure (MAP) and heart rate (HR) from Repeatedly Bred (RB) and Virgin rats from volume loading and mean circulatory filling pressure (MCFP) studies.

Baseline	Volume loading		MCFP	
	RB (n=8)	Virgin (n=7)	RB (n=6)	Virgin (n=6)
MAP mmHg	97.8±1.6	102.9±3.1	98.6±2.8	97.5±2.1
HR Beats/min	351.4±9.3	389.6±8.5	410.2±11.9	411.6±10.0

Date represented as mean ± standard error of mean.

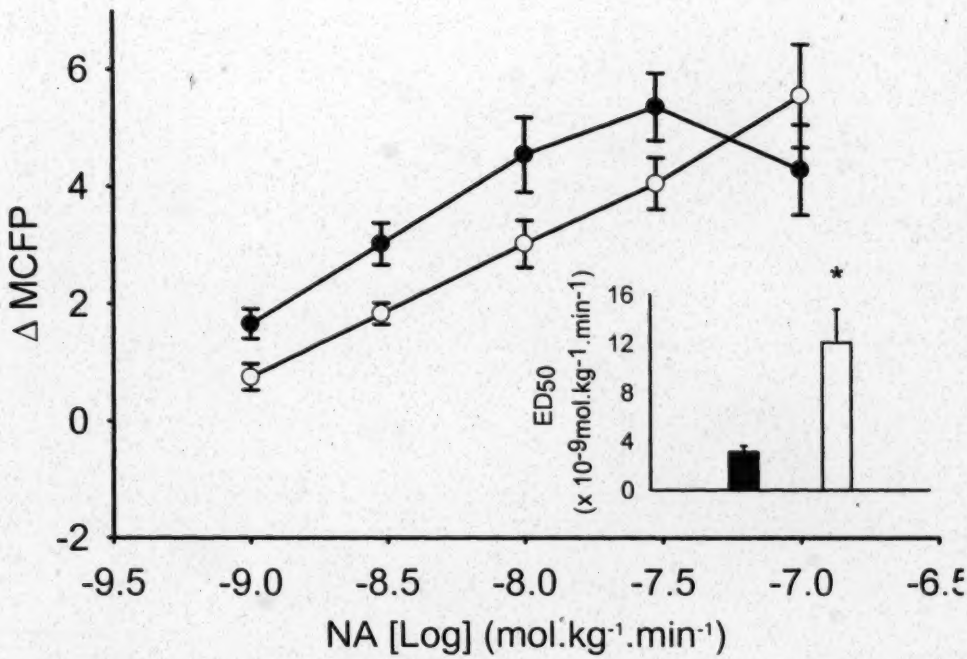

Figure 18: Effect of repeated pregnancy on changes in mean circulatory filling pressure (MCFP) responses to noradrenaline (NA) in conscious rats and MCFP ED_{50} response to NA. Repeatedly Bred rats: closed circles and bar (n=6); Virgins: open circles and bar (n=6).Vertical lines delineate standard error of mean.

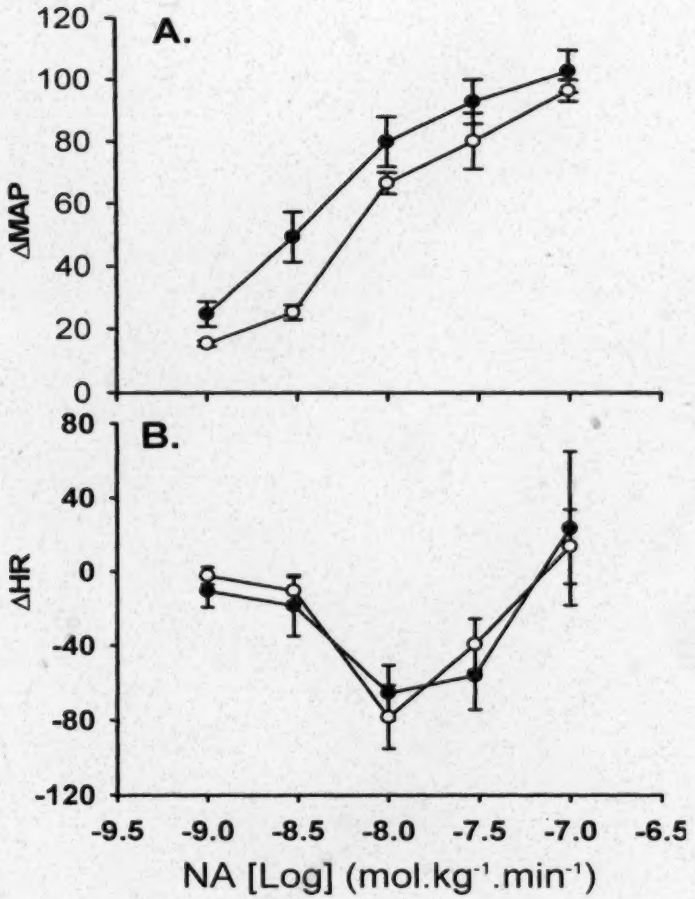

Figure 19: Effect of repeated pregnancy on changes in (A) mean blood pressure (MAP) and (B) heart rate (HR) responses to noradrenaline (NA) in conscious rats. Repeatedly Bred rats: closed circles (n=6); Virgins: open circles (n=6).Vertical lines delineate standard error of mean.

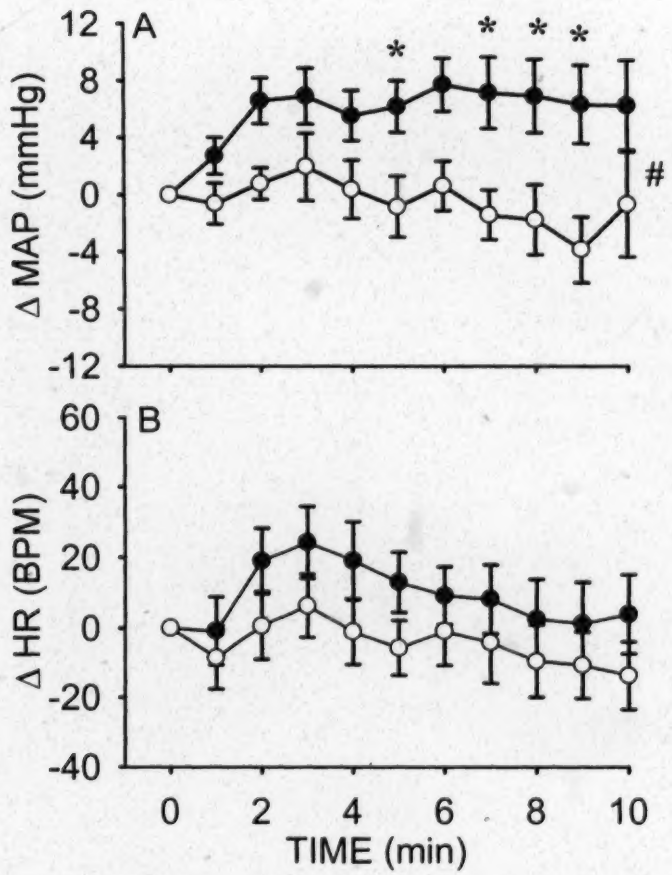

Figure 20: Effect of repeated pregnancy on changes in (A) mean arterial pressure (MAP) and (B) heart rate (HR) responses to volume loading (2mL/100g BWt) in conscious rats. Repeatedly Bred rats: closed circles (n=8); Virgins: open circles (n=7).Vertical lines delineate standard error of mean. #: $P<0.05$, significant difference between Repeatedly Bred and Virgin rats. *: $P<0.05$, individual points of significance between groups. **: $P<0.05$, significant increase relative to baseline value.

3.2.3 Effects of parity on reactivity and compliance of isolated veins:

3.2.3.1 Mesenteric venous reactivity and compliance:

Experiment A: Effects of parity on mesenteric venous reactivity:

Mesenteric Noradrenalin caused a dose-dependent decrease in venous diameter in both RB and Virgin rats (Figure 21); however the EC_{50} was lower in RB (2.68 $\pm 0.37\times10^{-8}$ M, n=5) than in age-matched Virgin control rats (EC_{50}: 4.67 $\pm 0.93\times10^{-8}$ M, n=8) ($P<0.05$). There was no significant difference in the baseline diameter (RB: 563.87±15.40 µm, n=5, Virgin: 568.6±14.79 µm, n=8, P=0.83) or maximum response (RB: 129.80±15.44 µm, n=5, Virgin: 127.375±29.05 µm, n=8, P=0.87) of veins from RB and Virgin rats.

Experiment B: Effects of parity on mesenteric venous compliance and capacity:

At physiological pressures (4-8 mmHg), the compliance (change in diameter/change in pressure) of veins was significantly lower in RB than in the age-matched Virgin rats (RB: 29.3±1.84 µm/mm.Hg, n=6 vs. Virgin: 36.95±1.34 µm/mm.Hg, n=6; $P\leq0.05$) (Figure 22). Maximum capacity of the isolated mesenteric veins (12mmHg) was lower in the RB rats (Figure 22). Wall to lumen ratio of veins from RB, measured at 6mmHg of transmural pressure, was also significantly greater than that from Virgin rats (RB: 0.247±0.02, n=6 vs. Virgin: 0.203±0.01, n=6; $P<0.05$). There was no significant difference in the stress-strain relationship of veins from RB and Virgin rats.

3.2.3.2 Effects of parity on saphenous venous reactivity: There was no significant difference between the sensitivity of saphenous veins from RB compared with those from Virgin rats (Figure 23).

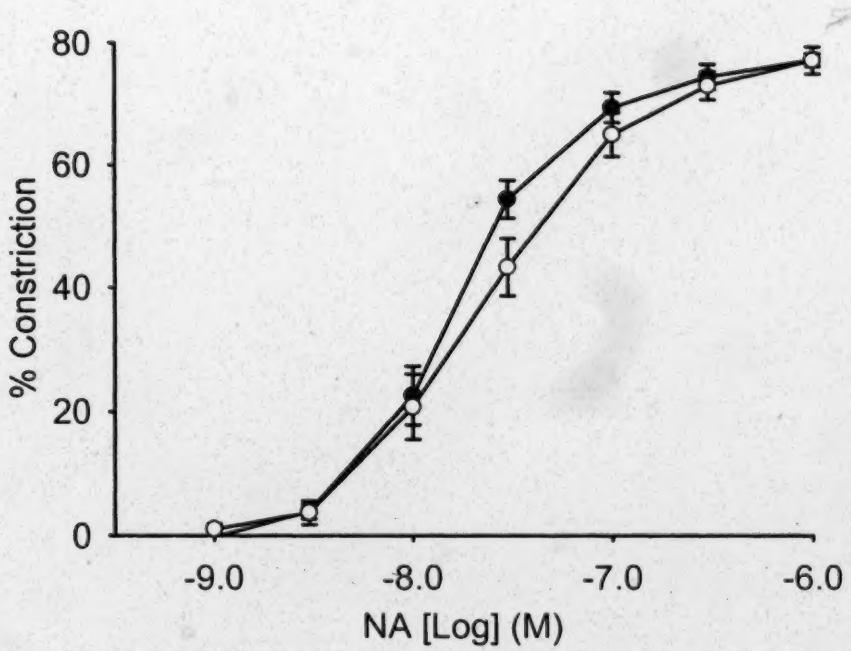

Figure 21: Concentration-response curves of noradrenaline (NA) on small mesenteric veins of Repeatedly Bred rats: closed circles (n=5); Virgins: open circles (n=8). Change in constriction is expressed as percentage of initial diameter. Vertical lines delineate standard error of mean.

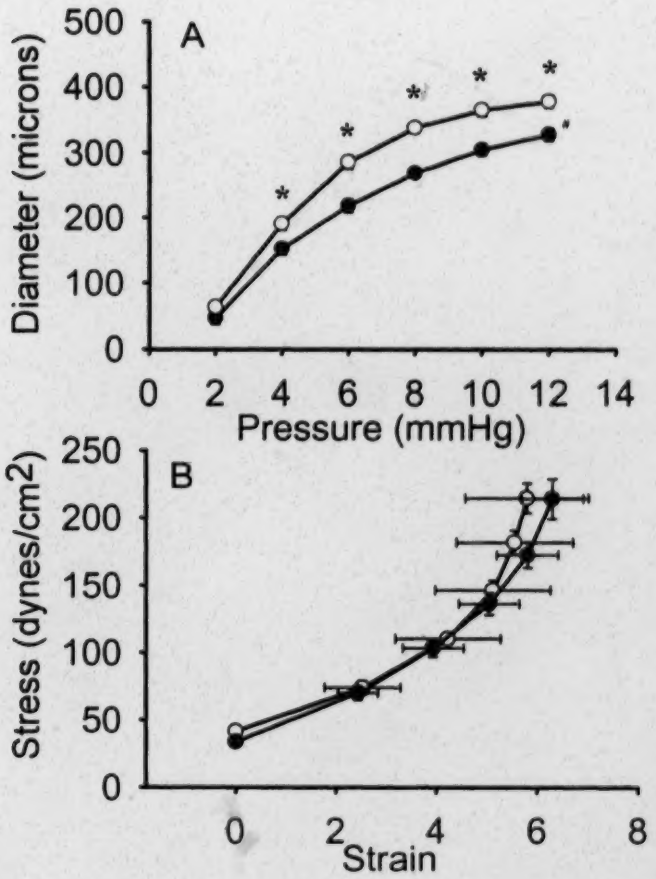

Figure 22: Effect of parity on change in A) diameter of small mesenteric veins as a function of pressure and B) stress-strain relationship in calcium free medium. Repeatedly Bred (closed circles, n=6) and Virgin rats (open circles, n=6). Vertical lines delineate standard error of mean. #: $P<0.05$, significant difference between Repeatedly Bred and Virgin rats. *: $P<0.05$, individual points of significance between groups. (Experiments conducted with the help of Dr. Zoë Brookes).

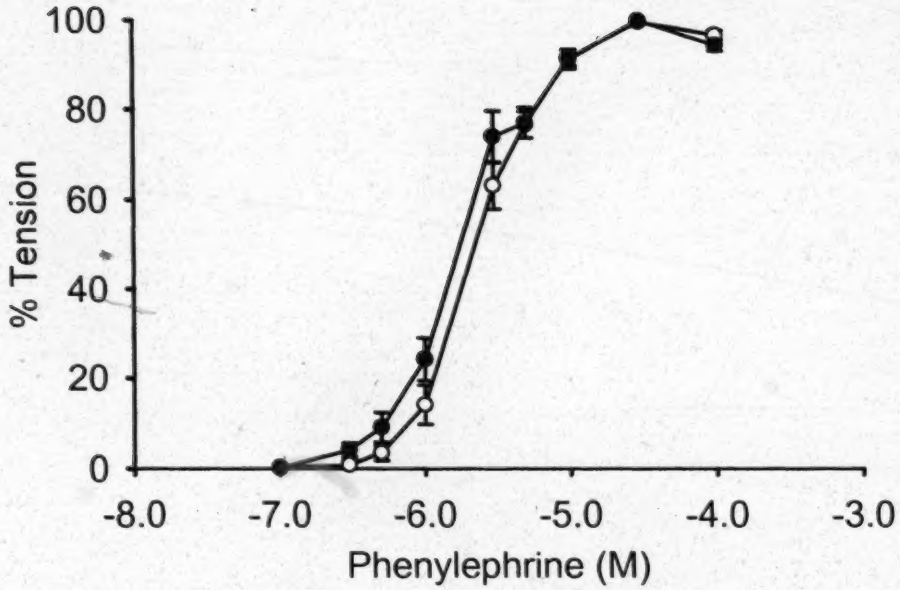

Figure 23: Concentration-response curves of phenylephrine (PE) on saphenous vein from Repeatedly Bred (closed circles, n=8) and Virgin (open circles, n=7) rats. Change in the tension is expressed as percentage of maximum response to PE. Vertical lines delineate standard error of mean.

3.3 Effects Of Parity On Serum Estrogen Levels:

There was no difference in the serum estradiol levels between the RB and Virgin rats (Figure 24; Median values: RB: 18.82 pg/ml, n=16 vs. Virgins: 15.59 pg/ml, n=15).

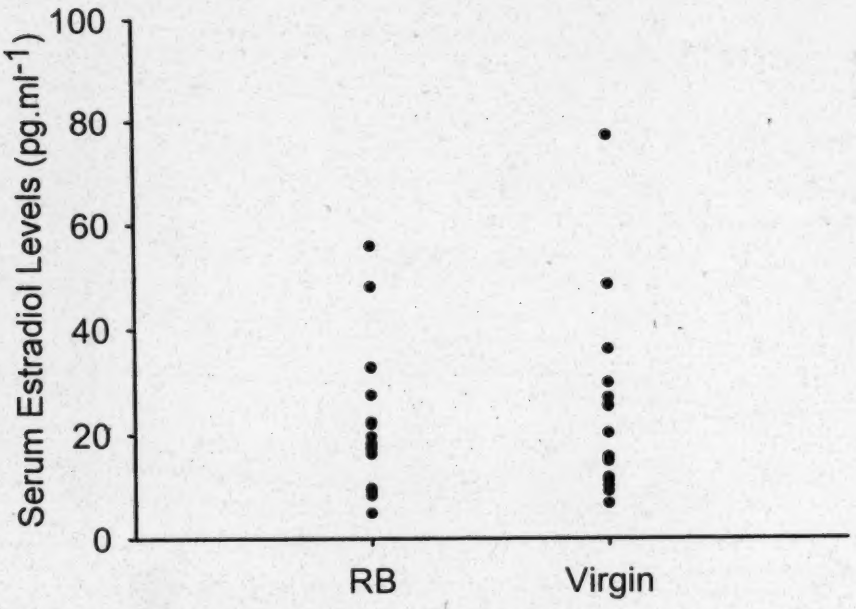

Figure 24: Serum estradiol levels of Repeatedly Bred (RB; n=16) and Virgin (n=15) rats.

4 DISCUSSION

4.1 Effects Of Parity On The Arterial System:

4.1.1 Effects of parity on pressor response to PE and acute stress:

This *in-vivo* experiment was designed to compare the baseline cardiovascular parameters, MAP and HR, between RB and Virgin rats. This study also investigated the difference between the pressor responses to both exogenous (PE) and endogenous (acute stress) stimulation of the adrenergic nervous system in the two groups. RB rats responded to exogenous PE and to acute stress with a greater increase in blood pressure than did the Virgin animals.

PE, a selective α1-adrenomimetic acting primarily on the blood vessels, causes a dose-dependent increase in MAP during intravenous infusion (91). PE is thus an excellent tool with which to investigate *in-vivo* vascular reactivity in the whole animal. The pressor response to PE infusion was greater in RB than in the Virgin rats. This difference was apparent in both the systolic (SBP) and diastolic (DBP) pressure traces, suggesting that the augmented pressor response to exogenous PE could be attributed primarily to increased TPR rather than to increased cardiac output (125,189). The magnitude of the pressor response to exogenous PE depends, not only on the sensitivity to vasoconstriction of the arterial resistance vessels (increase in TPR), but also on the buffering ability of the baroreflex system. We found no difference in baroreflex gain (ΔHR/ΔBP) between the RB's and the Virgins. This further supports our contention that the

greater pressor response of the parous animals was caused by a greater increase in TRP in the RB due to an enhanced sensitivity of their vasculature to PE. Our *in-vitro* studies confirmed this: Resistance-sized mesenteric arteries from parous rats had a greater sensitivity of constriction to PE in endothelium-intact mesenteric arteries than those from Virgin animals.

Stress induces a sympathetically mediated cardiovascular response which consists of an increase in MAP, HR, cardiac contractility and CO (122,223). Stress also causes the release of adrenaline from adrenal glands which activates β_1-adrenergic receptors which further increases HR (124,218). It must be noted that in conditions such as stress and exercise, the baroreflex is overridden by higher cardiovascular centers (such as hypothalamus) (89). This allows a simultaneous increase in HR and CO despite the increased MAP. Furthermore, resistance of various vascular beds is differentially affected (89). The resistance of mesenteric vascular bed is increased whereas that of skeletal muscles is reduced. This leads to redistribution of the blood to the skeletal muscle and central nervous system, an important component of Cannon's fight and flight reaction to acute stress. In rats, it has been reported that the increase in mesenteric resistance is primarily mediated through the activation of the sympathetic system, and that the decrease in resistance of the skeletal muscle vascular bed is due to sympathetic reduction/withdrawal (16,89).

Chronologically, both the MAP and HR responses in Figure 10A and B can be divided into 3 phases. In the initial phase, 0-5 sec after initiation of

stress, there was an increase in MAP accompanied by a reduction in HR; the increase in MAP is mediated by an increase in sympathetic stimulation to the vessels whereas the reduction in heart rate may be attributed to initial activation of the baroreflex. During this phase, it is the parasympathetic wing of the baroreflex system (which has been shown to take ~5 sec to initiate) that causes a depressor effect on the heart (n. vagus). Furthermore, this initial reduction in HR can also be attributed to the cardiac depressor reflex activated by the effect of air jet on the animal's face. In the second phase, 5-15 sec from the initiation of stress, MAP further increases, and is accompanied by an increase in HR. During this phase the increase in MAP is mediated by an increase in resistance of the splanchnic vascular bed and an increase in preload and cardiac output. We found that during this phase of the stress response, there was a greater increase in MAP in RB than in Virgin rats. This potentiated MAP response observed in RB could have been mediated either by a greater increase in splanchnic resistance or by an accentuated CO, or combination of both. In this experiment we did not measure CO. However, given that there was no difference in the HR response between the RB and Virgin rats (Figure 10B) and given that the stroke volume did not change, we can assume that CO was similar in both the groups. Furthermore, the potentiated pressor response to acute stress was associated with accentuated SBP and DBP responses, again suggesting that the augmented pressor response in the RB rats was mediated primarily through a greater increase in vascular resistance in this group.

In the third phase of the pressor response, 15-25 sec after initiation of stress, there was a reduction in MAP, although it still remained higher than baseline. This decrease in MAP was observed despite an increased HR. It is well know that during stress there is also activation of adrenal glands which releases adrenaline. Adrenaline is a non-specific adrenergic agonist which thorough activation of β2- receptors present on the skeletal muscle blood vessels, would reduce the tone of this vascular bed, thus reducing TPR. This would lead to the reduction in MAP observed during the third phase.

In RB, baseline MAP tended to be lower than that in Virgin rats, although this did not reach significance. A lower resting blood pressure in RB was also reported by Reckelhoff (168). By contrast, Baylis found resting MAP to be higher in RB rats (8). Given our finding that the pressor response to stress is greater in parous rats, this discrepancy may be attributed to the status of the animal during the experiment e.g. depth of anesthesia, anesthetic agent, and fluid/electrolyte balance. It was for this reason that we chose to measure blood pressure in fully recovered, unrestrained, conscious rats.

In this study, we have shown that there is a potentiated pressor response to both exogenous (PE) and endogenous (acute stress) stimulation of sympathetic nervous system. In both situations the increase in MAP was associated with a similar potentiated response in the DBP response in RB. Furthermore, there was no significant difference in the baroreflex sensitivity between the two groups.

This suggested that the increased pressor response in RB is due to a greater increase in the TPR.

TPR can be increased due to augmented activation of sympathetic system or due to increased vascular reactivity to adrenergic stimulation. Alterations in sympathetic activation and vascular reactivity can be, in part, attributed to changes in the hormonal environment. It has been reported that repeated pregnancy reduces the plasma level of estrogen (12), a hormone which has marked cardiovascular effects (5,12,57). Estrogen, through its genomic and non-genomic pathways, attenuates vascular reactivity to vasoconstrictors such as NA and angiotensin II (170,175,227). *In-vivo*, estrogen also attenuates the pressor response to PE by modulating both sympathetic outflow and baroreflex sensitivity (85,86). Estrogen, through its central and peripheral actions, has also been reported to attenuate pressor response to acute stress (36,145). Therefore decreased levels of estrogen could lead to a potentiated increase in constrictor response in RB rats.

During pregnancy, there are also marked alterations in the cortisol levels in various species (14,108) including rats (222). Interestingly, Wexler *et al* reported that there is an increased adrenal steroid and catecholamine production in RB rats, long after the reproductive activity has ceased (101,212). It is well known that adrenal steroids potentiate adrenergic sensitivity in the vascular bed (47,220). We did not investigate the effects of cortisol in our animals. However, given that there is hyperactivity of the adrenal gland in RB, we propose that

the potentiated pressor response in RB may be, in part, attributed to alterations in the release of steroid hormones.

The following study was designed to investigate the effects of parity on the reactivity of resistance sized blood vessels. We chose to study mesenteric (from splanchnic vascular bed) and femoral (from skeletal muscle vascular bed) arteries due to their specific roles in cardiovascular homeostasis. The splanchnic and skeletal muscle vascular beds receive 24% and 20% respectively of the total cardiac output and play a significant role in determination the total peripheral resistance as well as blood distribution within the intravascular space (118). Thus, any changes in the active (vascular reactivity) and/or passive (vascular compliance) properties of these vascular bed would be expected to significantly affect overall cardiovascular homeostasis.

4.1.2 Effects of parity on mesenteric arterial reactivity and compliance:

Isolated mesenteric arteries from RB rats were more sensitive to PE than were those from Virgin animals. The constriction response of a blood vessel is generated by its VSMC layer, which can be modulated by the underlying endothelium. The difference in PE sensitivity of mesenteric arteries derived from RB and Virgin rats was abolished when the endothelium was mechanically removed from the arteries. There was also no significant difference in the maximum response to PE in endothelium denuded arteries from RB and Virgins, suggesting that the overall constriction ability of the VSMC was unaltered. Furthermore, we did not find any difference in the wall thickness of the arteries between RB and Virgin animals. These results indicate that the difference in PE sensitivity between RB and Virgin rats is endothelium-dependent.

In endothelium-intact vessels, α-adrenergic constriction can be modulated by the endothelium derived relaxing and constricting factors (203,219). Multiple mechanisms have been proposed for the release of endothelium derived relaxing factors in response to VSMC constriction. Boer *et al* found that PE induces NO release in pulmonary artery and that the inhibition of NOS increases PE-mediated vasoconstriction. They proposed that PE directly act on the endothelial α_1-adrenergic receptors to stimulate NO release (15). Tuttle *et al* reported that PE causes an increase in endothelial cell calcium and proposed the presence of α_1-

receptors on endothelium (196). These studies suggest that PE constriction may be modulated through a direct action of PE on endothelial cells. It has also been suggested that the constriction of VSMC can be modulated by the mechanical effects of VSMC on endothelium cells. For example, Sun *et al* reported that constriction of VSMC causes deformation of endothelial cells resulting in the release of NO. It has also been found that PE-induced changes in VSMC calcium can influence the production of NO in the endothelial cells (55). Dora *et al* proposed this to be due to the diffusion of calcium from VSMC to the underlying endothelial cells through myoendothelial gap junctions to stimulate NOS (55).

The above studies suggest that stimulation of α-adrenergic receptors in blood vessels increases endothelial cell intracellular calcium, which leads to an augmented NO production. NO then diffuses to the VSMC and, through the cGMP pathway, causes relaxation of VSMC (9). We found that the difference in PE sensitivity between RB and Virgin rats is endothelium-dependent. Hence, we propose that repeated pregnancy might alter the PE induced release of endothelium derived relaxing factors in RB which might, in part, potentiate PE reactivity in endothelium-intact vessels from RB.

To investigate the mechanisms by which PE sensitivity was increased in parous rats, we blocked these pathways with L-NAME and meclofenamate respectively. L-NAME caused a significant increase in PE sensitivity of the mesenteric arteries in the Virgin rats, but not in parous rats, so that there was no longer any difference between the two groups. Furthermore, incubation of

mesenteric arteries with meclofenamate significantly shifted the PE concentration response curve to the right in RB but not in Virgin rats. These data suggest that repeated pregnancy blunts the adrenergic stimulated activation of the NO system, as well as enhancing the production of vasoconstrictive prostaglandins. Increased intracellular calcium (during endothelium activation) has also been shown to stimulate endothelium production of superoxide ions (203). Superoxide ions are scavenged by NO. Depending upon the bioavailability of NO, intracellular concentration of superoxide ions would increase which, in turn, would stimulate cyclo-oxygenase to convert arachidonic acid into vasoconstrictive prostaglandins (202,203). Prostaglandins would then diffuse to the VSMC and modulate VSMC constriction.

Several mechanisms could lead to a parity-induced decrease in the bioavailability of NO and an increase in vasoconstrictive prostaglandins. Pregnancy is associated with an increase in low density lipoprotein (LDL), total cholesterol and triglyceride levels (88,98). This imbalance in lipid profile persists postpartum (98). LDL and specifically the small dense LDL subfraction are highly susceptible to oxidation (158). It has also been reported that oxidized lipids can cause a decrease in eNOS mRNA and enzyme activity in human endothelium cells (123), thus decreasing the bioavailability of NO. Oxidized LDL can directly increase the production of super oxide ions and can also (33) recruit macrophages to generate superoxide ions (43,158,206). Superoxide ions are scavenged by NO to form peroxynitrite, which not only reduces the activity and expression of

eNOS (113,228) but also stimulates PGHS activity to generate vasoconstrictive prostaglandin endoperoxide and thromboxane. Superoxide ions can also, through conversion to hydroxyl radicals, lead to an increased production of vasoconstrictor prostaglandins (PGH_2), thus increasing the vascular constriction response (202). This is consistent with our finding that the vessels from parous rats were more sensitive to PE.

It has also been proposed that a decrease in NO production by the endothelium increases membrane fluidity. This leads to deceleration and trapping of the blood elements in microcirculation, which can lead to an increase in TPR (70). This is consistent with our *in-vivo* study that there was a greater increase in pressor response to adrenergic stimulation in the RB rats.

Interestingly, we did not find any significant difference in endothelium-dependent *vasorelaxation* of the RB and Virgin rats. This suggests that parity primarily alters vasoconstriction through modulation of adrenergic-dependent NO and cyclooxygenase pathways, rather than by altering endothelium-dependent vasodilatation per se. Increased vasoconstriction and attenuation of NO production have previously been reported in the renal vasculature of RB rats (168). Indeed, Reckelhoff has suggested that pregnancy might leave the vessels with some degree of endothelium damage which could ultimately cause endothelium dysfunction (168). Reckelhoff also proposed that pregnancy, which is a state of accentuated NO production in maternal body, can subsequently lead to attenuated production of NO through several negative feedback

mechanisms. Although the negative feedback mechanisms were reported in acute experiments, it would be interesting to determine their role in long-term reduction of NO. By contrast, Baylis and Rennke found no evidence for functional or structural abnormalities in the renal vasculature (8). Reckelhoff attributed this to a species-dependent difference in the effect of aging on the renal vasculature.

We found that a rise in intraluminal pressure caused a smaller increase in the passive diameter of small arteries from RB rats compared with those from Virgins i.e. they were less compliant. Furthermore, in RB, we found that the stress-strain relationship was shifted to the left. This indicate that for a particular stress (force experienced per unit of the tissue in the vascular wall), the accompanied changes in diameter was less in RB. This is consistent with previous reports of degradation of vascular elastic tissue in RB female rats (210-213). Changes in passive mechanical responses of a blood vessel depend upon the vascular elements such as smooth muscle content, medial thickness and collagen and elastin content of the vessels. It has also been suggested that not only changes in the concentration of collagen and elastin would alter vascular mechanical responses but also orientation of fiber and architecture of the vessel matrix. We did not find any difference in the maximum response to PE and in the wall thickness of the vessels derived from RB and virgin animals, suggesting that changes in collagen and elastin may be responsible for the decreased compliance in the arteries derived from RB. It has been suggested that increased collagen

synthesis may be the primary event in the development of the atherosclerosis observed in repeatedly bred female rats (211). The underlying mechanisms responsible for these changes in mechanics remain to be elucidated. Changes in hormonal environment have been shown to induce vascular remodeling (225). Estrogen and estrogen receptors levels correlate with a lower collagen concentration, indicating that estrogen through activation of estrogen receptor alpha protects against vascular collagen accumulation making the vessel more distensible (129). Increased oxidative stress has been shown to reduce vascular distensibility (67). Furthermore, pregnancy is a state of increased blood volume (157). Repeated volume overload, induced by repeated pregnancy, would cause the blood vessels to stretch and may potentially induce stretch injury. This would cause fracture, thinning and weakening of elastic fibers, leading to transfer of load to collagen. Collagen, being a stiffer extra cellular matrix protein than elastin, might lead to a reduced vascular compliance in RB (93,162,226). This decrease in vascular compliance would make the vessels stiffer and the cardiovascular system less able to compensate for any fluctuations in blood pressure. Furthermore, augmented reactivity to α-adrenomimetics would enhance the response to an increase in sympathetic outflow. This is consistent with our findings from the *in-vivo* studies. It should be noted that the passive compliance was studied in EGTA mixed calcium free-buffer. Although calcium free medium would have minimized the influx of extra-cellular calcium into VSMC and EGTA would have further scavenged any traces of extra-cellular calcium, there is a possibility that some

intra-cellular calcium still remained in the VSMC. Therefore this intracellular calcium potentially might have contributed to difference in passive compliance observed in RB and Virgin rats.

This study has shown that parity potentiates endothelium-dependent PE sensitivity in mesenteric arteries from RB due, in part, to modulation in the NO and cyclooxygenase pathways. We also found that mesenteric arteries from RB are less compliant than those from Virgin rats. Any changes in the active (vascular reactivity) and/or passive (vascular compliance) properties of the splanchnic blood vessels would be expected to significantly affect overall cardiovascular homeostasis, which would be consistent with our finding that there is potentiated pressor responses to PE and acute stress in RB.

4.1.3 Effects of parity on histology of aorta and mesenteric and renal arteries:

This study was designed to investigate both gross and microscopic differences in mesenteric, renal arteries as well as in the aorta. There were no gross morphological changes in either RB or Virgin rats, nor did we find any microscopic differences in them. This is in contrast to the findings of Wexler *et al.* where parous rats were reported to have an increased incidence (number and severity) of atherosclerotic plaques, and to have degenerative changes in vascular elastic tissues and decreased collagen content (209,211,212). Our histological studies also failed to observe any microscopic atherosclerotic changes in either RB or Virgin rats. However, the high incidence of atherosclerosis in the rats used in Wexler's studies may be linked to the pathogen status of those rats. There is increasing evidence that infectious agents can play an important role in development of atherosclerosis and can intensify the effects of other risk factors for the progression of atherosclerosis (62,140). Most of the previous studies examining the relationship between parity and atherosclerosis were carried out 40-50 years ago, when laboratory rats were not pathogen free. By contrast, the rats used for these current experiments were specific pathogens free (SPF rats from Charles River, St Foy, Quebec, Canada). This might explain the reason why none of our rats had any signs of atherosclerotic lesions. This does also raise the question as to whether, if parous individuals are exposed to risk factors (such as

infectious agents), that might contribute to their having a higher probability of developing cardiovascular disease than non-parous individuals.

4.2 Effects Of Parity On Venous System:

4.2.1 Effects of parity on venous tone response to NA (MCFP Study):

Given that there are significant alterations in the venous vasculature *during pregnancy* (93-95,187), we investigated the long-term effects of repeated pregnancy on venous tone. We measured MCFP, an important determinant of venous return to the heart and cardiac output, and an index of venous tone (165). We found no difference in baseline MCFP or baseline MAP and HR. However, there was a potentiated MCFP response (reduced ED_{50} response) to NA infusion in the RB rats, suggesting that repeated pregnancy causes an increased venoconstrictive response to sympathetic stimulation. In MCFP dose response curve it appear that the Virgin group has not yet reached its maximum response, however no further dose of NA was administered as the MAP had already reached a significantly high value.

NA infusion also caused a greater increase in MAP in RB compared to Virgin rats, which is consistent with our previous findings of a greater pressor response to PE in RB. Given that β-adrenergic receptors were blocked by propranolol before NA infusion during MCFP measurements, the increased pressor response to NA in RB rats must have been predominantly due to increased constriction to α-adrenergic receptor stimulation. There was no significant difference in the baroreflex sensitivity of the two groups. This is consistent

with our previous findings that RB rats have a greater pressor response to physical stress and to PE infusion (54). Given that propranolol would have blocked the β-adrenergic cardiac receptors, the reflex tachycardia observed during NA infusion can be attributed predominantly to parasympathetic withdrawal and not to sympathetic activation. This observed tachycardia could also be due to the pharmacological properties of propranolol. Propranolol is a competitive β-blocker. Potentially, at higher doses of NA, NA could have successfully competed with propranolol for cardiac β_1-adrenergic receptor and induced tachycardia.

One might have expected that the baseline MCFP determined in Experiment 3.2.1 should have been elevated. However, the technical limitations of the protocol to measure MCFP may have precluded our being able to detect such a difference. The experiments had to be done just a few hours after surgery in order to minimize the risk of a thromboembolytic event. However, at this time, resting sympathetic tone would undoubtedly have still been elevated in both the RB and Virgin animals, which may have masked any potential difference in basal sympathetic control of splanchnic venous tone.

MCFP is the mean vascular pressure in the cardiovascular system when the circulation is stopped (166). MCFP represents the upstream venous pressure that drives venous return (79). MCFP is proportional to venous tone and stressed volume. In the absence of changes in blood volume an increase in MCFP response denotes an increase in venoconstriction and/or a reduction in venous compliance (165). In our study, we measured MCFP responses to NA in the absence of

hemorrhage or infusion of fluid. Therefore we can assume that the blood volume in our animals was not altered and the changes in MCFP responses to NA could be attributed to increase in venous tone. Increased venous tone could be due to increased sympathetic outflow or due to alterations at the postjunctional levels. To further explore the mechanism underlying the mechanisms behind the greater increase in venous tone during NA infusion in parous animals, we studied the venous reactivity and compliance of mesenteric and saphenous veins.

The MCFP findings suggest that, during sympathetic stimulation, the venous system in RB rats would exhibit higher tone than would that of Virgin rats. This would cause increased venous return and, ultimately, increased cardiac preload (73,166). The resultant increase in cardiac output would then contribute to a potentiated pressor response to sympathetic stimulation in RB.

4.2.2 Effects of parity on blood volume and blood pressure response to volume loading:

During pregnancy venous compliance and tone undergo tremendous changes (97). These changes allow for accommodation of the increased blood volume, and maintain the increased cardiac output required during pregnancy. The long-term effects of repeated episodes of these alterations on the cardiovascular system are unknown. The present study was designed to investigate the effects of repeated pregnancies on the competency of the cardiovascular system to buffer volume loading, and to compare the blood volume of RB and Virgin rats. The data reveal that RB responded with a greater pressor response to volume loading than did Virgin rats. Furthermore, the total blood volume in RB was lower than that in Virgin rats.

Our finding that comparable volume loads induced a greater increase in blood pressure in RB than in Virgin rats, suggests that RB are less able to buffer increases in intravascular volume than are Virgin rats. However, during volume loading, instead of a reflex bradycardia in response to volume loading there was an increase in HR. The *increase* in HR, rather than the anticipated reflex bradycardia, can probably be attributed to the Bainbridge reflex, whereby intravenous volume loading can increase HR (84). Although there was no significant difference between the two groups, there was a significant increase in HR in the RB rats (at 3 min), but not in the Virgins. This again is consistent with

their having an impaired ability to accommodate the volume load in the splanchnic circulation, so that there was a greater transient increase in cardiac preload.

An increased pressor and HR response to volume loading and an increased MCFP response to NA suggest that, under conditions both of tonic autonomic withdrawal and of stimulation, total body venous tone would be higher in parous animals. Consistent with this was our finding that, even under basal conditions, blood volume was lower in the RB rats. Although it might be argued that obesity can reduce the ratio between blood volume and body weight (177), our animals had been maintained on a reduced calorie diet to prevent obesity (107). The difference in body weight between our parous and nulliparous animals was thus minimal, and was unlikely to have contributed to the reduced blood volume in the RB rats.

The venous side of the splanchnic vascular bed plays a critical role in the homeostatic responses to changes in intravascular volume (179,197). The splanchnic venous system is highly compliant and contains approximately 30% of the total circulating blood. Furthermore, its intraluminal volume can be actively modulated by both neural and circulating factors in the blood (80,161). This part of the venous system is thus important in cardiovascular regulation. Our attention was therefore directed to whether there is a difference between the reactivity and compliance of isolated splanchnic (mesenteric) veins derived from RB and Virgin rats.

4.2.3 Effects of parity on reactivity and compliance of isolated veins:

Given that the venous side of the splanchnic vascular bed plays a critical role in regulating cardiac output and blood pressure (179,197) and that there was an augmented MCFP and pressor response to noradrenaline and to volume loading respectively, we tested the hypothesis that repeated pregnancy increases NA sensitivity and reduces venous compliance in mesenteric vessels from RB rats. We also studied the reactivity of saphenous vein in RB and virgin rats. Saphenous vein represents venous bed of skeletal muscle, one of the largest vascular beds in circulation. These veins also undergo profound alterations during pregnancy and, in humans, these alterations remain postpartum. Therefore we were interested in investigating the long term effects of repeated pregnancy on both saphenous and femoral venous systems.

We found that the EC_{50} of NA of isolated mesenteric veins from RB rats was significantly lower than that for vessels from Virgins. There was however no difference in the maximum constriction response to NA. Thus parity predominantly potentiates venous sensitivity to NA, while constrictive capacity is unaltered. These data suggest that, during sympathetic stimulation, the splanchnic vascular bed would exhibit higher venous tone in RB rats than in Virgin rats, which would augment venous return and cause transient increases in cardiac preload (73,166).

Whole body venous tone, as well as vascular reactivity of isolated veins, may be modulated by NO (35,68,104). Given the evidence that repeated pregnancy reduces arterial endothelial NO (54,168), the increased *venous* responsiveness of the RB rats to NA may probably also be attributed to reduced bioavailability of NO.

There is also some evidence that NO may modulate vascular compliance (111). We found that a rise in intraluminal pressure caused a smaller increase in the diameter of veins from parous rats compared with those from Virgins i.e. they were less compliant (210,213). However there was no difference in the stress-strain relationship of mesenteric veins. Reduction in compliance and lack of changes in stress-strain relationship may be due the increase in the wall: lumen ratio observed in RB. We did not find any difference in the maximum contraction of the veins derived from RB and Virgin animals, suggesting that the increase in the wall: lumen ratio in RB may be associated with alterations in elastin and collagen content. This decrease in mesenteric venous compliance would make the splanchnic vascular bed less distensible, and the cardiovascular system would be less able to buffer any fluctuations in blood volume.

The *in-vitro* venous responses to NA were potentiated in the parous rats. Although the question may arise as to how changes in vasoreactivity of isolated venous segments may relate to the control of overall splanchnic tone, it should be pointed out that these results are consistent with our *in-vivo* experiments showing an augmented NA-induced increase in MCFP in the RB rats. Furthermore,

blood volume in the RB rats was significantly lower, which may also be attributed to higher sympathetic vascular tone (11). This concurrence between our *in-vitro* and *in-vivo* results lends credence to our contention that there is indeed a difference between the two groups with regard to reflex control of splanchnic vascular capacitance.

Studies of the changes in venous reactivity and compliance during pregnancy have been difficult to interpret. With regard to whole body compliance, there has been evidence for there being an increase (52), a decrease (99), or no change (30). Although Humphreys & Joels reported a pregnancy-induced increase in MCFP which was independent of sympathetic tone, they found total body compliance to be increased (97). Part of the difficulty in evaluating these results arises from the fact that both the splanchnic and peripheral vascular beds contribute to whole body compliance, and that the degree to which each contributes probably varies according to the experimental conditions (anesthesia, species etc). Whereas compliance of the limb veins increases and reactivity decreases during pregnancy (115,187), mesenteric venous reactivity increases and compliance decreases (92,94,95). This reflects the very different functional roles of these vascular beds, the splanchnic circulation being important not only in delivering blood to the tissues, but also in controlling blood distribution and cardiac preload (73,80,161). Focusing, as we did on the splanchnic circulation, we found mesenteric venous reactivity to be higher and compliance lower in the parous rats than in the Virgins. This suggests that, even long after pregnancy,

the splanchnic venous circulation retains many of the characteristics acquired during pregnancy (92,94,95).

The mechanisms underlying the effect of repeated pregnancies on venous tone and compliance are uncertain. It has been reported that pregnancy permanently alters levels of several reproductive hormones which have potent vascular activity. For example, repeated pregnancy reduces plasma levels of estrogen (12). Through its genomic and non-genomic effects, estrogen increases the production of NO, attenuates the pressor response to vasoconstrictors and increases vascular compliance i.e. it acts as a vaso-protective agent (5,17,175,180,205). Considering the abundant localization of estrogen receptors in the circulatory system (90), we investigated the serum levels of estrogen in RB and Virgin rats.

4.3 Effects Of Parity On Serum Estrogen Levels:

It has been reported that, in humans, pregnancy permanently reduces plasma levels of estrogen (12). Estrogen has well-established effects on the cardiovascular system. It has been shown to increase NO biosynthesis, to attenuate arterial vasoconstriction to PE, angiotensin, and vasopressin, and to increase vascular compliance (5,65,207). Estrogen has also been shown to attenuate *in-vivo* pressor response to PE infusion through it action on the baroreflex sensitivity and on resistance sized blood vessels (86). However, we did not find any difference in the serum estradiol levels between RB and Virgin rats. Although plasma levels were similar, we cannot discount the role of this hormone entirely, since repeated pregnancy has also been reported to significantly reduce the population of estrogen receptor positive cells, at least in the mammary glands (193,221). It would therefore be interesting to investigate the population of estrogen receptors in parous animals.

5 CONCLUSIÓN

Based on the results presented in this thesis and their interpretation in the discussion the following conclusions are made:

1. Parity causes a potentiated pressor response to both endogenous (acute stress) and exogenous (PE) stimulation of sympathetic system. Based on the systolic and DBP responses and lack of change in the baroreflex sensitivity, this appears to be due to a potentiated increase in vasoconstriction in RB rats.

2. Parity causes an increase in PE sensitivity of mesenteric arteries, which was endothelium-dependent. Both NO and cyclooxygenase pathways play a role in parity-induced modulation of PE sensitivity. We propose that this potentiated sensitivity to α-adrenergic agonist in parous rats contributes to the increased pressor response to PE in these animals.

3. The reduced compliance and increased sensitivity to NA in mesenteric veins during pregnancy persists long after reproductive activity has ceased.

4. Repeated pregnancy augments the pressor response to volume loading. There is also an augmented increase in total body venous tone in response to α-adrenergic receptor activation. This is due, at least in part, to changes in venous compliance and venous reactivity to NA in the splanchnic vascular bed.

5. Repeated pregnancy does not cause any alteration in the serum estrogen levels.

It is difficult in epidemiological studies to control for potentially confounding factors such as socio-economic status and the psychological stresses of child rearing (155). Thus, well-controlled animal studies are critically important. In Rats, not only are the pregnancy-associated changes very similar to those found in humans (183), but also repeated pregnancies can be induced within a short interval of time.

Figure 25 provides a general overview of the proposed mechanism of cardiovascular modulation due to parity. In the rat, we have shown that repeated pregnancy augments the pressor response to intravenous infusion of vasoconstrictors (PE and NA) and to acute stress due, at least in part, to changes in both the passive and active characteristics of the mesenteric blood vessels. Both NO and cyclooxygenase pathways play a role in these alterations, changes which could lead to transient stress-induced increases in TPR and cardiac afterload. Furthermore, repeated pregnancy induces a long-term reduction in splanchnic venous compliance, and augments splanchnic venous reactivity. This compromises the ability of the capacitance (venous) system to accommodate volume overloads and to buffer changes in cardiac preload. These transient changes in afterload and preload could, in time, contribute to the increased risk for cardiovascular disease observed in multiparous individuals.

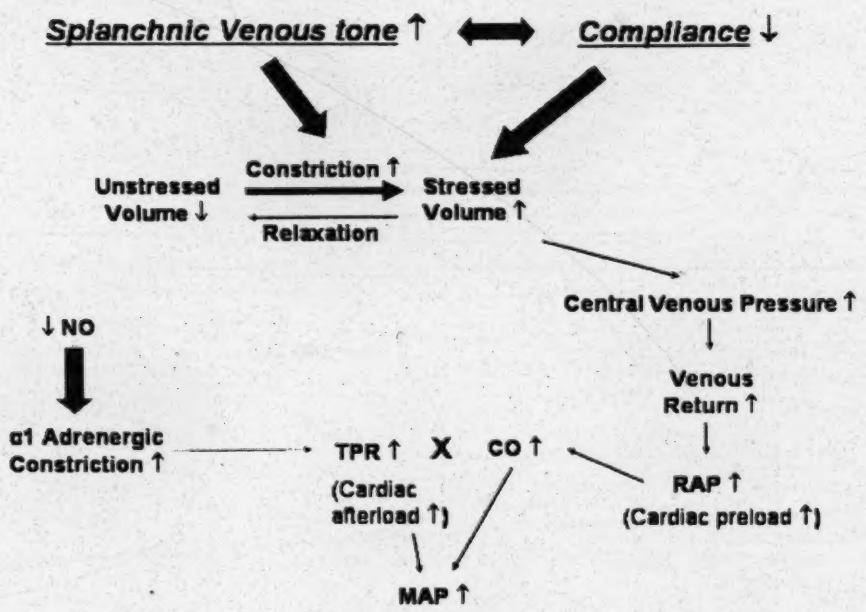

Figure 25: Representation of mechanism of cardiovascular modulation due to parity. RAP: Right Atrium Pressure, CO: Cardiac Output, TPR: Total Peripheral Resistance, MAP: Mean Arterial Pressure, NO: Nitric Oxide.

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7. APPENDIX A

Isometric Pressure Myography: A Novel Method To Study Vascular Reactivity.

ABSTRACT

Evaluation of vascular function of isolated blood vessels is critical to understanding cardiovascular pathologies. Currently, isobaric pressure myograph and isometric wire myograph systems are used to examine vascular reactivity. However, there are certain limitations associated with both these systems; although the pressure myograph allows one to investigate a vessel under more physiological conditions than the wire myograph, it is not possible in pressure myography to measure a true maximal response. This is because, at higher concentrations of a vasoconstrictive factor, the lumen of the vessel may be obliterated, precluding evaluation of the constrictive responses to higher levels of the factor under investigation. The goal of the present study was to evaluate a technique which could circumvent this problem and allow for characterization of the full dose-response curve. This was achieved by subjecting pressurized vessels to isometric conditions. Using a video dimension analyzer and a diameter servo controller, we maintained the cannulated isolated vessels at a constant diameter and recorded the vascular responses to PE in terms of changes in intraluminal pressure. The sensitivity of this system was assessed using prazosin. Concentration-response curves from the isometric pressure myograph-system were similar to those from the isobaric-pressure myograph system. Furthermore, isometric pressure myography was able to distinguish the response of PE in control and prazosin treated vessels. We propose that isometric pressure

myography offers distinct advantages over other methods for constructing concentration response curves of isolated vessels.

INTRODUCTION

One of the major patho-physiological mechanisms underlying cardiovascular disease is alterations in reactivity resistance sized vessels (2,5). It is therefore critically important to study the vascular function of these vessels. Since the introduction of the wire- (3) and pressure myograph (10) systems to study the functional properties of isolated vessels, there has been considerable growth of knowledge in this area of vascular pathology.

Both the isometric wire- and isobaric pressure myograph are excellent tools to study vascular function. Using the isometric wire myograph, isolated vessels are exposed to a certain diameter (isometric condition) and their responses to various vasoactive agents are measured in terms of the change in wall tension. In this system, the vessel is mounted on two parallel wires, which prevents the vessel wall from moving during contraction. This allows the determination of force generated by the vessel walls through a force transducer connected to one of the wires. Because of its isometric conditions, this system also allows one to measure the maximum response of a vessel to a vasoconstrictive agent. It is also useful in experiments where the vessel walls must be held steady, including electrophysiological setups where the electrodes need to be at a constant distance from the vessel walls. However, because the vessels mounted on the wire myograph system are stretched using two wires, there is always a risk of damaging the endothelium. In addition, the vessels acquire a distorted and "non-

physiological" shape.

In the isobaric pressure myograph, isolated vessels are cannulated and exposed to a certain pressure (isobaric condition), during which they maintain their normal physiological form. The pressure myograph is therefore considered to be a physiologically more appropriate tool to study vessel function. However, there are some limitations associated with this system, primarily that it is unable to determine the maximum response of a vessel to a vasoactive agent. This is because, at higher concentrations of a vasoconstrictive factor, the lumen of the vessel may be obliterated, precluding evaluation of the constrictive responses to higher levels of the factor under investigation. Furthermore, because the vessel diameter is not held constant during contraction, its intra-luminal volume changes. This may result in an intraluminal flow of perfusate which may cause sheer stress on the endothelium cells, and alter the vascular response.

The goal of this study was to develop a technique, which could eliminate some of the disadvantages of both the isobaric pressure- and isometric wire myograph systems. This was achieved through development of an isometric pressure myograph system, where pressurized vessels were subjected to isometric conditions. Using a video dimension analyzer and a diameter servo controller, we maintained the cannulated isolated vessels at a constant diameter and recorded the vascular responses to PE. The sensitivity of this technique was assessed using prazosin, a selective α1-adrenergic blocker. Concentration response curves from isometric pressure myograph-system were similar to those from isobaric-

pressure myograph system. Furthermore, the isometric pressure myograph was able to distinguish the response of PE in control and prazosin treated vessels. We propose that this system offers distinct advantages over other methods for generating concentration response curves of isolated vessels.

MATERIALS AND METHODS

The experimental procedures were approved by the local Animal Welfare Committee in accordance with the guidelines issued by the Canada Council on Animal Care, which conforms to NIH guidelines.

Animal and Housing: Male Long Evans rats were obtained from Charles River, St Foy, Quebec, Canada. A period of at least 1 week from arrival was allowed to elapse before the experiments were started, during which time the rats were held in the University of Alberta animal facility on a 12-h–12-h light dark cycle, in a humidity and temperature-controlled environment.

Drugs and Solution: All the vascular reactivity experiments were conducted in HEPES-buffered phosphate saline solution (HEPES-PSS); composition (in mmol/L): 142 NaCl, 4.7 KCl, 1.17 $MgSO_4$, 1.56 $CaCl_2$, 1.18 K_2PO_4, 10 HEPES and 5.5 glucose. A pH of 7.4 was then achieved by adding NaOH solution. Stock solution of PE, prazosin, noradrenaline were prepared in distilled water and stored at -40°C. Immediately before the experiments, the drugs were thawed and dissolved in HEPES-PSS to achieve the required concentration. All the drugs and salts were purchased from Sigma Aldrich (Canada).

Surgical Procedure: The rats were sacrificed by decapitation. The distal part of the small intestine and its associated vascular arcade were rapidly removed through a midline laparotomy and transferred to a silicone coated dissecting dish containing ice-cold HEPES-buffered phosphate saline solution (HEPES-PSS).

Second-order segments of mesenteric arteries were dissected free from surrounding adipose tissue, cut into ~2-mm lengths, and transferred to either an isobaric pressure myograph, an isometric pressure myograph or an isometric wire myograph.

Pressure myograph System:

A schematic of the myograph used in this study is shown in Figure 26. Two-way switches S1 and S2 were simultaneously turned to alternate the setup between setup A (isobaric pressure myograph setup) and setup B (isometric pressure myograph setup). In setting A, the inflow cannula was connected to a pressure servo system (PS200/Q, Living Systems Instrumentation, Burlington, VT) through a pressure transducer. This pressure servo system maintained a constant intraluminal pressure using a peristaltic flow pump. Vessels were viewed with a black and white CCD camera (Hitachi, Canada) and displayed on a monitor (Ultrak). Internal vessel diameter (ID) was measured using a video dimension analyzer (V94, Living Systems Instrumentation, Burlington, VT). Both internal vessel diameter (ID) and intraluminal pressures were recorded online using WINDAQ data acquisition software (Windaq, DATAQ Instruments, Akron, OH). In setup B, the switches S1 and S2 were turned to position B, which set the system to isometric pressure myograph mode. Vessels were viewed with the CCD camera and the ID was measured using the video dimension analyzer. Signals from the vessel dimension analyzer were fed to the diameter servo, which maintained a constant vessel diameter by changing the intraluminal pressure using a

peristaltic pump. Thus, as soon as the internal diameter of the vessel would start to decrease in response to a constrictive agent, intraluminal pressure would be increased to maintain the initial vessel diameter, and vice versa. The changes in pressure were measured using a pressure transducer attached to the inflow cannula. The pressure signals were then amplified (Gould Pressure Transducer, Model: 13.4615.50, Gould Inc. Cleveland, OH), digitized (DI-205, Dataq Instruments, Akron, OH) and recorded online using WINDAQ data acquisition software.

Isobaric pressure myography:

The pressure myograph system (CH/2/SH; Living systems, Burlington, VT) was adjusted to setup A (isobaric pressure myography). Isolated vessels were transferred to the vessel chamber which contained ice-cold HEPES-PSS. The vessels were then mounted on the inflow cannula (diameter 100-125 μm) and secured with a single fiber of a multifilament braided nylon thread. At this point the inflow stopcock was opened, and a gentle flow was allowed through the lumen of the vessel, keeping pressure <5 mmHg for 1 minute to flush the blood and metabolites from the lumen. Flow was then stopped and the distal end of the vessel was mounted and tied onto the outflow pipette. The flow was again started for 1 minute to ensure the continuity of the system. The distal stopcock was then closed so that blind-sac (no-flow) experiments could be performed. After mounting the vessels in a small-vessel chamber, intraluminal pressure was briefly (10 seconds) increased to 80 mmHg to ensure there were no leaks in the

system; the system was considered to be leak-free if there was no drop in pressure during this time. The vessel chamber was slowly warmed to 37°C and maintained at 37 ± 0.5°C throughout the duration of the experiment. The intra-luminal pressure was increased to 60mmHg and the vessel was allowed to stabilize for 30 min in HEPES-PSS buffer, during which time the buffer solution was changed at 10-min intervals. To ensure the vessel did not buckle at the higher concentrations of PE, intraluminal pressure was increased 160mmhg after the third washout, and the vessel was stretched just enough to restore its form After stabilization, concentration response curve to PE were generated. Vessels were viewed with a black and white CCD camera (Hitachi, Canada) and displayed on a monitor (Ultrak). Internal vessel diameter (ID) and wall thickness were measured using a video dimension analyzer (Living Systems Instrumentation). Both ID and intraluminal pressures were recorded for later off-line computerized analysis using WINDAQ (DATAQ Instruments).

Isometric pressure myography:

The myograph system was initially adjusted setup A (isobaric pressure myography). Isolated vessels were mounted on two glass cannulae as described above. The intra-luminal pressure was increased to 60mmHg and the vessels were allowed to stabilize for 30 min in HEPES-PSS buffer, during which time the buffer solution was changed at 10-min intervals. To ensure the vessel did not buckle at the higher concentrations of PE, intraluminal pressure was increased 160mmhg after the third washout, and the vessel was stretched just enough to

restore its form. The preparation was then allowed to stabilize for further 10 min at 60 mmHg, after which the baseline internal diameter was recorded. The myograph was then switched to setup B (isometric pressure myography), the HEPES-PSS buffer was changed, and the vessel was allowed to stabilize for 5 minutes. PE concentration response curves were then generated, where changes in pressure as a function of PE concentration was measured. To measure the sensitivity of this system other sets of vessels were treated with 10^{-8} or 10^{-6} M prazosin for 20 min, after which PE concentration response curves were generated.

Isometric wire myography:

Isolated mesenteric arteries were threaded on two 25 μm diameter tungsten wires (Fine wire company, California, USA) and were then mounted on an isometric small wire myograph station (Kent Scientific, Litchfield, California).

Protocol: After mounting, vessels were allowed to stabilize for 30 min in HEPES-PSS buffer under zero tension, during which time the buffer solution was changed at 10-min intervals. Preconditioning stretch was performed, and the vessels were then allowed to stabilize in HEPES-PSS buffer for another 10 min. This was followed by generation of a baseline curve to determine resting length-tension property according to the method described Halpern *et al.* (12). From Laplace's law, the circumference that an artery would have at a transmural pressure of 100 mmHg (L_{100}) was calculated from the exponential curve fit of tension

generated vs. internal vessel circumference. Preliminary studies on second order mesenteric arteries taken from rats indicated that the point on the passive-active tension characteristics curve obtained at $0.8L_{100}$ provided the maximum active tension, with the least passive tension. The vessels were then set at their optimal tensions and concentration response curves to PE were generated. Real-time changes in isometric force were recorded on a computer using data-acquisition software (Windaq, DATAQ Instruments, Akron, OH).

STATISTICAL ANALYSIS

Concentration-response curves were plotted and the EC_{50} and Hill slope were calculated using SigmaPlot software (Systat Software Inc., USA). For simple comparisons between two sets of data such as EC_{50}, slope and baseline intraluminal pressure, we used Students t-test. In Figure 3, the difference between control and prazosin treated (10^{-8}M) vessels was assessed using repeated measures two-way ANOVA, followed by post hoc analysis with the Student-Newmans-Keuls test. All Data are presented as mean $\pm$ SE of mean. All results were considered statistically significant at $P<0.05$.

RESULTS

Isometric pressure myograph: The mean baseline intraluminal pressures at which the vessels were stabilized were as follows: Control: 59.56±0.15 mmHg, n=5; prazosin 10^{-8}M: 60.51±0.586 mmHg, n=6; prazosin 10^{-6}M: 61.72±1.21 mmHg, n=3). Figure 27 shows the average concentration-pressure curve to PE in isometric pressurized vessels. To a stepwise increase in the concentration of PE, there was an increase in the intraluminal pressure which was needed to maintain the vessel diameter constant.

To study the sensitivity of the isometric pressure myograph system we measured the concentration-pressure curves to PE in the presence of the competitive and selective α1-adrenoblocker, prazosin. Figure 27 illustrates that the potency of PE was significantly reduced with prazosin at a concentration of 10^{-8}M and was completely abolished with a concentration of 10^{-6} M. The PE EC_{50}, slope and maximum response of concentration-pressure curve of the control vessels and vessel treated with prazosin are shown in Table 5. The EC_{50} of the control vessels was significantly lower than that of vessels treated with prazosin (10^{-8}M). However, there was no significant difference between the Hill slopes of the two curves. There was also no significant difference between the maximum responses of the control vessels and the vessels treated with prazosin (10^{-8}M).

Isobaric pressure myography: Under isobaric conditions, there was a decrease in the diameter of the vessels exposed to PE (Figure 28). The PE EC_{50}, the

concentration of a PE required to induce 50% of the maximum response, calculated from the concentration-response curves generated from the vessels mounted on the isometric pressure myograph system, was not significantly different from that of the vessels mounted on the isobaric pressure myograph system.

Isometric wire myography: In mesenteric arteries, an increase in PE concentration produced an increase in tension (Figure 29). However, the PE EC_{50} value of the vessels mounted on isometric wire myograph ($20.43\pm2.02\times10^{-6}$M, n=7) was significantly higher than that of the vessels mounted on the isometric pressure myograph ($0.44\pm0.67\times10^{-6}$M, n=5) ($P\leq0.001$).

DISCUSSION

We evaluated the characteristics of isometric pressure myography in the study of reactivity of small mesenteric arteries. In the isometric pressure myograph technique, the diameter of the vessel was kept constant with a diameter servo control system. The pharmacological responses to constricting agents were then measured in terms of changes in pressure which were required to counter the force produced by the constriction of the vessel. We found that the sensitivity of isolated mesenteric arteries mounted on the isometric pressure myograph was not significantly different from that of the vessels mounted on isobaric pressure myograph system. However, vessels mounted on the pressure myograph, isometric or isobaric, were far more sensitive to PE than were those mounted on the isometric-wire myograph system.

Bevan *et al.* in 1970 introduced the isometric wire myograph technique; which was one of the first standardized techniques to the study function of small isolated vessels (3). Using this technique, the vascular response to a vasoactive agent is measured in terms of change in isometric tension (12). Wire myography is an excellent tool for studying vascular pharmacology; it precisely describes the functional properties of blood vessels by measuring EC_{50}, slope and the maximum response of a concentration-response curve (4). However, because the vessels are mounted on two wires and are stretched, they acquire a distorted and "non-physiological" shape (7). To overcome this problem the isobaric pressure

myography, was developed. In the isobaric pressure myograph system, isolated vessels are mounted on two cannulae and are then exposed to a certain pressure (isobaric condition). This allows the vessel response to be measured in terms of changes in vessel diameter (1,9).

Compared to isometric wire myography, the isobaric pressurized-system has some obvious advantages (6,9), and is thus considered to provide a more physiological assessment of vessel function. In pressurized vessels the endothelium of the blood vessels is not touched with wires and there is therefore no damage to this important regulator of vascular tone (8). Furthermore, because the vessels are pressurized, they are held in their normal shape as opposed to the vessels mounted on a wire myograph, where the stretched vessels acquire an elliptical shape (7). However, there are two major limitations to isobaric pressure myograph system. First, at higher concentrations, the constrictive agents cause vasoconstriction to such an extent that the lumen of the vessel is completely obliterated (unpublished observations). This interferes with the ability to measure any further changes in vascular tone, and renders the technique incapable of measuring the maximum vasoconstriction response. Maximum constriction response is an important determinant of the overall constriction capability of blood vessels, and is known to be altered in disease processes such as hypertension. Furthermore, accurate determination of the maximum response is required to calculate the EC_{50} of a concentration response curve (4), a parameter which is critical to characterizing vascular function. Secondly, because the

vessel diameter changes upon vasoconstriction, there is some degree of uncontrolled intraluminal flow generated during the vasoconstriction response. It is well established that flow is a major stimulus for one of the most potent endothelium-dependent vasodilators, NO (14). Therefore, it is logical to suggest that the vascular response measured using isobaric pressure myography would be influenced by artifactual biosynthesis of NO.

In the isometric pressure myograph system, the pharmacological responses to vasoactive agents are measured in terms of change in intraluminal pressure; the vessel diameter is kept constant by means of a diameter servo control system. This allows the vessel to preserve its normal physiological shape, without disturbing the vessel endothelium. In contrast to isobaric pressure myograph, this technique also allows one to determine the maximum response to the vasoactive agent. Moreover, the isometric condition of this system allows only minimal changes in intraluminal volume compared with the isobaric pressure myograph system. Although we did not directly measure changes in the flow in either the isobaric or isometric pressure myograph systems during vasoconstrictive responses, it is apparent that there would be only a minimal changes in the intraluminal flow in vessels mounted in the isometric system. This would significantly reduce the potential effects of flow-induced release of NO on the vascular response. It has also been reported that vascular constriction (change in vessel diameter) is accompanied with endothelium deformation, which may stimulate NO release, another mechanism which could potentially modulate

vascular response to constrictive agents in the isobaric pressure myograph (13).

Concentration-response curves of the isolated mesenteric vessels were generated in the absence and presence of prazosin (10^{-8}M) to test the sensitivity of the isometric pressure myograph system. Prazosin is a selective and competitive α1-adrenergic blocker. In the presence of prazosin (10^{-8}M), there was a significant increase in the EC_{50} and a right-ward parallel shift in the concentration-response curve of the vessels compared those from control vessels, without altering the maximum responses of the vessels from two groups. At a much higher concentration of prazosin (10^{-6}M), the effect of PE was completely abolished.

As reported earlier (7), the pressurized vessels were significantly more sensitive to adrenomimetics compared with vessels mounted on an isometric wire myograph. Wire mounted and pressurized vessels have a different geometrical shape (7) and are under different longitudinal stretch (6). These conditions modulate amine reuptake in the blood vessels, and have been reported to be responsible for observed decreased adrenomimetic sensitivity of the wire mounted vessels (7).

In the vessels studied using the isometric-pressure myograph system it is possible that the changes in intraluminal pressure in response to a vasoconstrictive agent could modulate the concentration response curves. Bevan *et al.* reported that cannulated mesenteric vessels exposed to pressure of 20-60 mmHg had different norepinephrine sensitivities (6). They suggested that stretch and/or

pressure could to be responsible for these differences. It has also been reported that in cannulated vessels an increase in transluminal pressure causes an increase in intracellular Ca^{2+}, which may influence the smooth muscle cell response (11). However, in both those studies, the increase in transluminal pressure also caused an increase in the internal diameter of the vessel (6,11) thus subjecting the vessel to a circular distention. Therefore it is not clear whether it was actually the transmural pressure or an increase in the vessel diameter which caused the changes in vascular reactivity.

In the present project we evaluated the feasibility of the isometric pressure myograph system. Concentration-response curves generated from the vessels mounted in the isometric pressure myograph-system were similar to those from the isobaric-pressure myograph system. In contrast to isobaric pressure myography, isometric pressure myography enables one to determine the maximum response to vasoconstrictors. We propose that this system offers advantages over the traditional isobaric myograph system for studying concentration response curves of small isolated blood vessels.

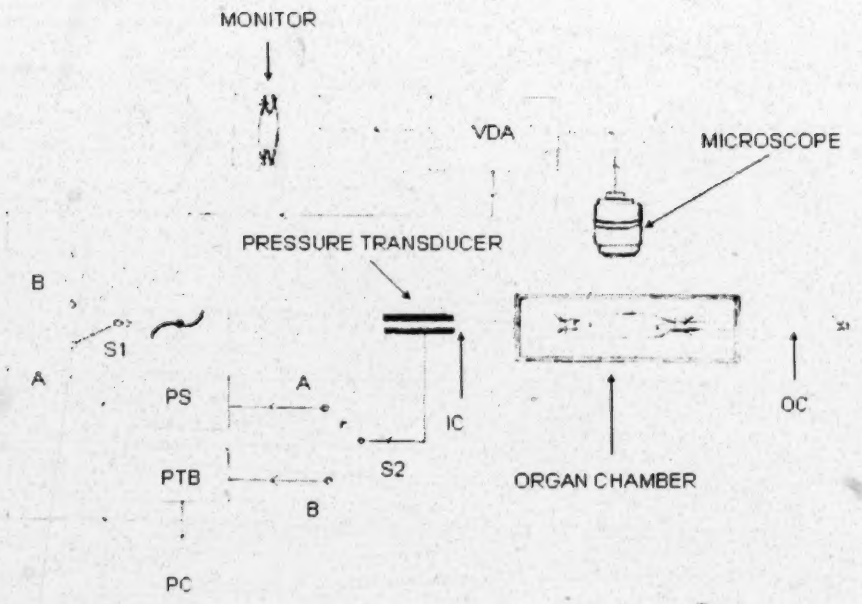

Figure 26: Schematic of isometric pressure myograph system. Isolated vessel is mounted on inflow (IC) and outflow (OC) cannula in an organ chamber. Intraluminal pressure is sensed by a pressure transducer. Vessel image is fed to video dimension analyzer (VDA) through a microscope and viewed on a monitor. The signal from the VDA is also fed to computer (PC) for recording internal diameter. Switches S1 and S2 are used to alternate the setting of this system between a pressure control servo (isobaric pressure myograph system, Setting A) and diameter control servo (isometric pressure myograph system, Setting B). PS, pressure servo box; PTB, pressure transducer box.

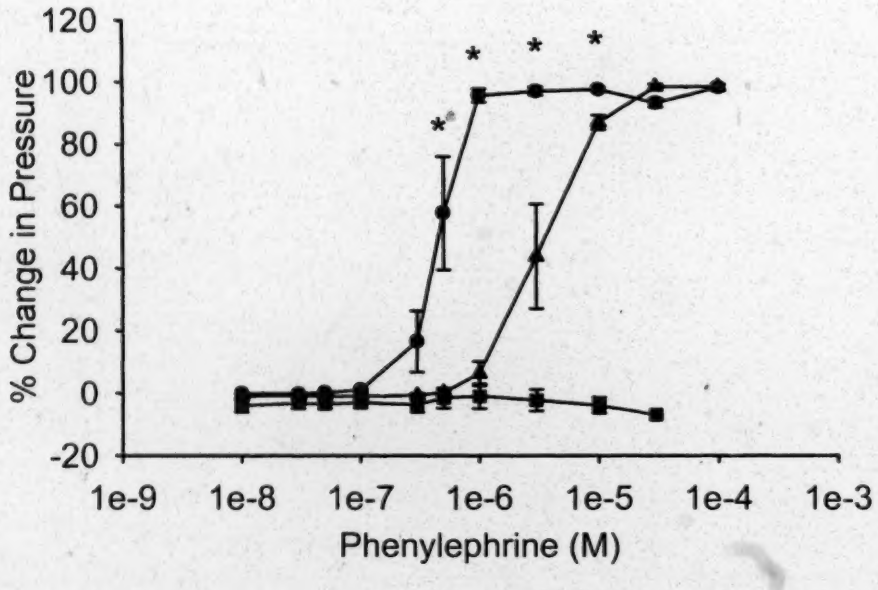

Figure 27: Concentration-response curves to phenylephrine (PE) of control (closed circles, n=5) and prazosin- (10^{-8} M, Closed triangles, n=6 and 10^{-6} M, closed squares, n=3) treated isolated mesenteric arteries mounted on isometric pressure myograph system. Changes in pressure are expressed as percentage of maximum response. Vertical lines delineate the standard of mean.

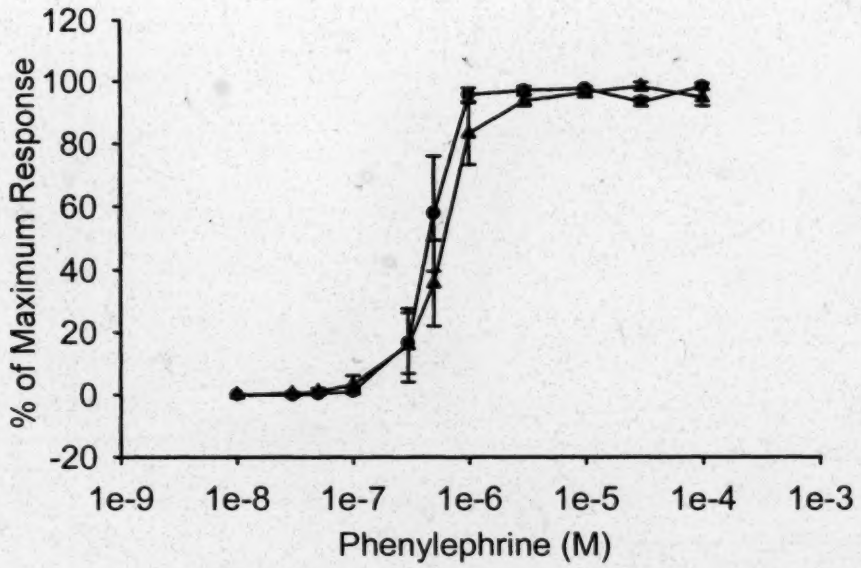

Figure 28: Concentration-response curve to phenylephrine (PE) of isolated mesenteric arteries mounted on isometric pressure myograph system (closed circles, n=5) and on isobaric pressure myograph system (closed triangles, n=4). Response from isometric pressure myograph system is expressed as percentage of maximum change in pressure and those from isometric wire myograph as percentage maximum change in vessel tension. Vertical lines delineate the standard of mean.

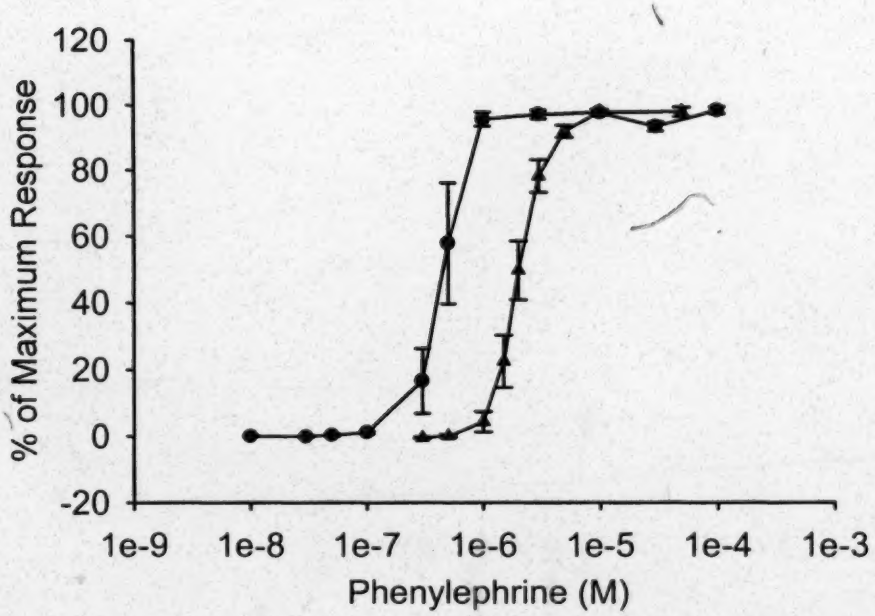

Figure 29: Concentration-response curve to phenylephrine (PE) of isolated mesenteric arteries mounted on isometric pressure myograph system (closed circles, n=5) and on isometric wire myograph system (closed triangles, n=4). Response from isometric pressure myograph system is expressed as the percentage of maximum change in pressure and those from isometric wire myograph as the percentage maximum change in vessel tension. Vertical lines delineate the standard of mean.

Table 5: Phenylephrine EC_{50}, slope and maximum response of control (n=5) and vessels incubated in 10^{-8}M prazosin (n=6).

	EC_{50} (10^{-6}M)	Hill Slope	Max Response (mmHg)
Control (n=5)	0.44 ± 0.06*	-12.10 ± 4.51	118.12 ± 4.21
Prazosin 10^{-8}M (n=6)	4.47 ± 1.25	-3.46 ± 0.35	126.82 ± 7.80

Data presented as mean ± standard error of mean. *, $P<0.05$, significant difference between control and prazosin treated vessels.

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APPENDIX B

Effects Of Parity On Venous Reactivity Studied Using Wire Myography.

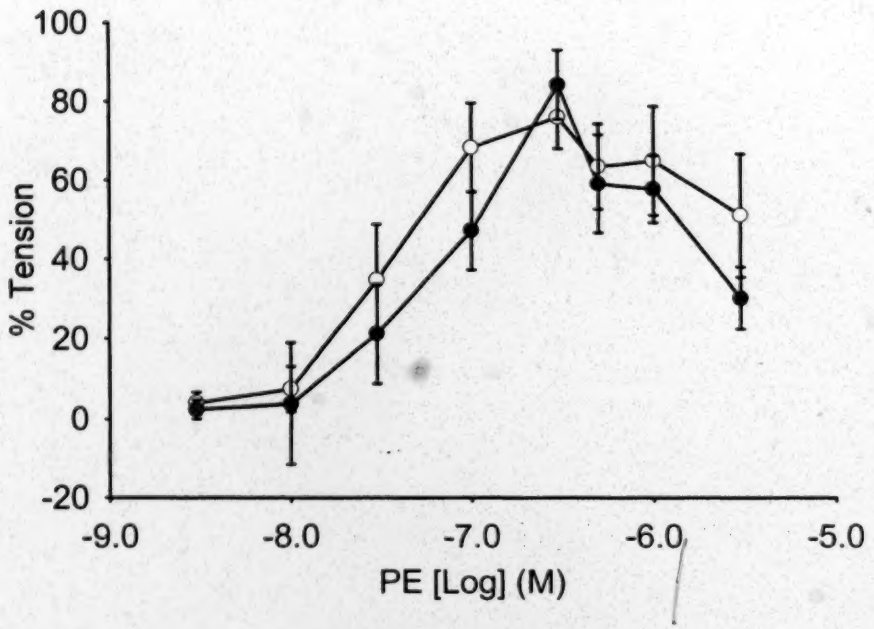

Figure 30: Concentration-response curves of phenylephrine (PE) on small mesenteric veins from Repeatedly Bred (closed circles, n=6) and Virgin (open circles, n=6) rats. Change in the tension is expressed as percentage of maximum response to PE. Vertical lines delineate standard error of mean.